Incorporating rhizosphere microbiota from the native and non-native ranges into tests of post-naturalisation performance: New Zealand *Trifolium* as a model system

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Abstract

Incorporating rhizosphere microbiota from the native and non-native ranges into tests of post-naturalisation performance: New Zealand *Trifolium* as a model system

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A critical goal in the study of plant invasions is to understand the traits and mechanisms that contribute to invader success. One of the most compelling hypotheses is the evolution of increased competitive ability (EICA), which posits that invasive plants escape co-evolved pathogens, pests and herbivores from their native range and adapt by down-regulating defences in favour of fitness, thus becoming superior competitors. However, after two decades of rigorous testing, support for all of EICA's predictions remains equivocal. This lack of consensus may exist because most tests have centred on aboveground interactions, omitting the interactive effects of rhizosphere microbiota, which play pivotal roles in plant performance, fitness and competitive ability. Furthermore, EICA investigations have focused solely on antagonists, while post-naturalisation trade-offs that result in increased competitive ability can also arise when plants are dislocated from mutualists.

In this thesis, I use *Trifolium* species as a model system and expand the EICA framework by growing plants from native (European) and non-native (New Zealand) provenances in rhizosphere soil cultivated *in situ* by conspecifics in each range. Using this biogeographical framework, I first compared the performance of plants from each provenance and analysed their association with root endophytes (arbuscular mycorrhizal fungi (AMF) and nitrogen-fixing rhizobia). Second, I compared profiles of root flavonoids (which function in both defence and mutualisms) in plants from each provenance when grown in sterilised or unsterilised rhizosphere soil, allowing me to separate microbe-stimulated versus constitutive production. Lastly, I performed an intra-specific competition experiment to test whether non-native plants have developed superior competitive ability. For each investigation, I predicted that the magnitude of divergence between plants from native and non-native provenances would be positively correlated with the naturalisation success of each species, which for these New Zealand *Trifolium* species is not correlated with their naturalisation date.

Non-native plants had lower rhizobia nodulation compared with native conspecifics, and this difference was significant in New Zealand and UK soils. AMF colonisation varied, but was significantly lower among non-natives in Spanish soil. Flavonoid richness was generally reduced among non-native plants—significantly in sterilised native-range soils (suggesting constitutive down-regulation), and in one of the native-range unsterilised soils (UK). However, there was no evidence for performance trade-offs; instead, non-native plants were significantly smaller than native conspecifics in all soils. Neither was there evidence of physiological compensation for decreased mutualist associations, nor increased competitive ability. Lastly, there were no correlations between the magnitude of trait divergence and species' distributions in the non-native range. Thus, despite significant post-naturalisation differences in a number of traits that might suggest adaptation, these differences do not appear correlated with increased plant performance nor with the naturalisation success of *Trifolium* in New Zealand.

This thesis contributes four important findings to our knowledge of post-naturalisation performance among invasive plants. First, phenotypic differences may be apparent between native and non-native conspecifics, but these differences do not necessarily equate to improved fitness. Second, mutualist availability and effectiveness may not necessarily be a substantial barrier to naturalisation—even among plants that host multiple highly beneficial symbionts in their native range or those that encounter parasitic mutualists in the introduced range. Third, the standard EICA metrics growth and competitive ability are not always the most relevant factors to indicate invasibility, as this study supports work showing decreased size may be equally common and successful invaders are not always better competitors. Fourth, this work revealed that measures of size are not an appropriate surrogate for measures of competitive ability—a valuable finding for future EICA experimental designs.

Keywords: Alien, arbuscular mycorrhizal fungi, competition, daidzein, defence, evolution of increased competitive ability, EICA, enemy release, exotic, HPLC, invasive, isoflavonoid, microbiome, mutualism, plant-soil interactions, rhizobia, rhizosphere, root flavonoid, symbiosis.

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To make a prairie it takes a clover and one bee,—
One clover, and a bee,
And revery.
The revery alone will do
If bees are few.

— Emily Dickinson, American poet (1830-86)

This work would not have been possible without the assistance of countless mentors, colleagues, friends and family.

I first wish to thank the Royal Society of New Zealand, which funded this ambitious project through a generous Marsden grant and enabled me to undertake international field work, expansive glasshouse experiments and costly chemical analyses. New Zealand has truly led the movement toward battling invasions, ferociously working to protect its endemic flora and fauna while suffering the highest rates of invasion in the world. I sincerely hope the findings of our project spur greater bio-protection of a country I have come to care for deeply.

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Declaration

The glasshouse experiments presented in Chapters 2-4 were designed and conducted in partnership with Kevin J. McGinn, a PhD student at Lincoln University also using the New Zealand *Trifolium* study system.

Parts of this thesis were submitted for peer-review publication prior to thesis submission. Co-authors were Philip Hulme, Wim van der Putten, Kevin McGinn, Carolin Weser and Richard Duncan. An earlier version of some of Chapter 4 was submitted to *Biological Invasions* (May 7, 2014), sent out for review and was not accepted for publication. The major shortcoming identified by the reviewer was the seed and soil collection regime "with 5 introduced populations (provenances in the authors jargon) in a small region of New Zealand and 5 locations in Spain and UK representing the native range (equally clustered and thus inappropriate)." It was felt that at least 10 populations across a climatic or latitudinal cline should be collected in each region. The manuscript was revised and submitted to *Oecologia* (Nov. 2, 2014), sent out for review and was not accepted. The primary criticism was that without detailed knowledge about enemies of the invasive plants in the native and non-native ranges it was not possible to explain the results thoroughly even if evidence for the EICA in New Zealand had been found. The manuscript was revised again and submitted to *AoB PLANTS*. It was accepted and published online March 10, 2016; doi: 10.1093/aobpla/plw016.

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Chapter 1

General Introduction

1.1 Adaptation as a mechanism for plant invasion

Globalised trade, travel and transport have accelerated anthropogenic dispersal of organisms around the world. Although only a small fraction of organisms survive relocation and even fewer naturalise outside their non-native ranges (Lockwood *et al* 2007), those that form self-sustaining, spreading and unwanted populations can severely degrade local ecosystems and cause major economic losses (Pimentel *et al.* 2001). A key goal in invasion ecology is therefore to understand which traits and mechanisms make invaders successful (Levine *et al.* 2003). Of the great number of hypotheses that have been proposed specifically to explain plant invasions (Stamp 2003; Joshi & Vrieling 2005; Liu & Stiling 2006; Diez *et al.* 2009; He *et al.* 2009; Sun & He 2010; Doorduin & Vrieling 2011; Lowry *et al.* 2012), one of the most prominent is the evolution of increased competitive ability (EICA), based on the premise of optimal defence theory. Early in the study of plant invasions, it was observed that plants that naturalise outside their native range often perform better—attaining greater size or fecundity, suffering less from pests and out-competing native flora (Darwin 1859; Elton 1958). The EICA hypothesis posits that the increased performance of invaders results from plants escaping co-evolved enemies from their native range and then redirecting energy away from the defences that protected them from these enemies and toward fitness, thus making them superior competitors (Blossey & Nötzold 1995).

The EICA hypothesis arose from a study of *Lythrum salicaria* (Lythraceae), a European native that is widely naturalised in North America where it out-competes native plants to such a degree that it forms dense monocultures that disrupt riparian ecosystems. Plants from a single population from each range were grown in a common garden. Reduced defence was deduced by the higher survival and greater body sizes of a root-feeding weevil on non-native plants compared to native conspecifics; increased competitive ability was inferred from non-native plants being larger than native conspecifics (Blossey & Nötzold 1995). Later EICA tests expanded this limited experimental design by incorporating more populations from each range (Willis & Blossey 1999), using multiple invasive genera (Blumenthal & Hufbauer 2007), comparing the release from specialist versus generalist herbivores (Abhilasha & Joshi 2009), directly measuring foliar defence compounds (Maron, Vilà & Arnason 2004b; Cipollini *et al.* 2005) and performing competition experiments with common heterospecifics in each range (McKenney *et al.* 2007) or with conspecifics from the native range (Bossdorf *et al.* 2004). Because the central prediction of EICA is that increased fitness co-occurs with

reduced defences, most tests reintroduce invaders to antagonistic biota from the native range to test whether they are more poorly defended than native conspecifics—the basis of contemporary biocontrol efforts (Hinz & Schwarzländer 2004; Muller-Scharer, Schaffner & Steinger 2004). Strong support for EICA has been found in several invasive taxa, notably *Sapium sebiferum* (Euphorbiaceae) (Siemann & Rogers 2001), *Spartina alterniflora* (Poaceae) (Daehler & Strong 1997), and *Buddleja davidii* (Scrophulariaceae) (Ebeling, Hensen & Auge 2008). The EICA phenomenon has also been tested experimentally outside the context of invasions: native *Oenothera biennis* (Onagraceae) rapidly adapted to insecticide-induced freedom from herbivory by down-regulating defensive ellagitannins in fruit and became more competitive (Agrawal, Hastings & Johnson 2012).

Two decades of rigorous EICA testing has provided compelling evidence for post-naturalisation adaptation, or at least shifts in performance traits that may contribute to plant invasions, however consistent support for all the predictions of the EICA hypothesis remains equivocal (Atwood & Meyerson 2011; Lowry *et al.* 2012; Felker-Quinn, Schweitzer & Bailey 2013). For example, some invaders increase defences (Abhilasha & Joshi 2009) or shift from producing specialist-targeted to general defence compounds (Muller-Scharer *et al.* 2004); some invaders are smaller in the non-native range (Buswell, Moles & Hartley 2011); and most tests fail to find evidence of superior competitive ability (Felker-Quinn *et al.* 2013). Lack of consensus may be due to EICA studies omitting a key factor that drives both plant defence and fitness—the role of rhizosphere microbiota (but see Volin *et al.* 2010). Microbial communities in the rhizosphere are the primary mediators of plant establishment and persistence (Revilla *et al.* 2012; Coats & Rumpho 2014) playing pivotal roles in plant performance and competitive ability (van der Putten & Peters 1997; Boyden, Binkley & Senock 2005; Sun & He 2010; Sabais *et al.* 2012) with important implications related to post-naturalisation shifts in the performance of non-native plants (Volin *et al.* 2010). Most tests of EICA have instead focussed on aboveground herbivory by macroinvertebrates, so plants from the native and non-native range are typically grown in a common garden with commercial potting mix or neutral soil (i.e. lacking the rhizosphere microbial communities associated with conspecifics in each range). These tests attempt to isolate the genotypic differences between plants from native and non-native provenances by comparing phenotypes under the same conditions (Colautti, Maron & Barrett 2009), but they do not incorporate the interactions taking place belowground—including how plant genotypes may respond differently to rhizosphere microbiota in the native versus the non-native ranges.

The central goal of this thesis is therefore to improve upon the standard EICA framework by incorporating rhizosphere microbial communities from both the native and non-native ranges into multi-species tests looking for evidence of post-naturalisation adaptation and increased competitive ability. Working from this biogeographical, belowground framework, I selected three areas of focus

to fill knowledge gaps. Specifically, I investigate the effects of: (i) loss of co-evolved mutualists (whereas EICA tests look only at the role of antagonists); (ii) down-regulation in root chemistry—both constitutive compounds and those synthesised in response to rhizosphere microbiota (whereas previous tests have focussed on foliar defences); and (iii) post-naturalisation competitive ability of plants measured using intra-specific pairings with conspecifics from the native range (whereas most tests use growth as a surrogate for competitive ability).

1.2 A belowground, biogeographical approach

Plants interact most intimately with biota in the "rhizosphere" (Hiltner 1904)—the biologically active soil region around roots that is rich with chemical signals and densely populated by microbiota, including bacteria, archaea, fungi, nematodes, oomycetes, tardigrades, paraphyletic eukaryotes (historically classed protists or protozoa), and root endophytes such as mycorrhizae and *Trichoderma*. Cumulatively, the rhizosphere microbiome has a profound influence on plant physiology and community composition (Philippot *et al.* 2013; Coats & Rumpho 2014)—both indirectly, by affecting soil structure, biogeochemical cycling and nutrient availability (Jeffries *et al.* 2003), and directly, as individual microbes can be positive or negative to plant growth (van der Putten, Klironomos & Wardle 2007). Rhizosphere communities also directly impact plant competitive ability—antagonistic microbes decrease fitness by stimulating the production of energetically costly defences, whereas beneficial microbes enhance fitness by making limiting nutrients more accessible or stimulating plant-growth-promoting hormones (Rout *et al.* 2013). Many soil microbes—both antagonistic and mutualistic—infiltrate and proliferate inside plant root tissues; typical bacterial loads can be 10^5 CFU/g fresh root weight (Schulz, Boyle & Siebert 2006). Root-rhizosphere microbiota interactions are dynamic; the composition and abundances of microbes both respond to and impact the composition of plant colonisers and these relationships measurably enhance or retard plant growth and fitness (Figure 1.1).

Rhizosphere microbial communities are intrinsically different among geographic locations (Pringle *et al.* 2009; Tedersoo *et al.* 2014); despite the early assumption that "everything is everywhere, but the environment selects" (Baas Becking 1934), biome profiling is showing that the vast majority of soil microbes are dispersal-limited (Andonian *et al.* 2012; Rout & Callaway 2012; Nuñez & Dickie 2013; Bardgett & van der Putten 2014; Tedersoo *et al.* 2014). Constraints to geographic relocation are especially strong for root endophytes as they are generally intolerant to variations in soil pH, temperature, soil minerals, salinity and moisture (Singh, Bhatt & Pant 2011; Nuñez & Dickie 2013). In addition, some microbial mutualists must be matched with a host plant of a particular species or even genotype (Bever, Westover & Antonovics 1997; Inderjit & van der Putten 2010; Rout & Callaway 2012) and those that spend part of their lifecycle outside the plant host (e.g. those that associate

with annual plants) must compete with other microbes in the soil matrix between plant generations (Gaur & Lowther 1982; Parker 1999; Rodríguez-Echeverría *et al.* 2012).

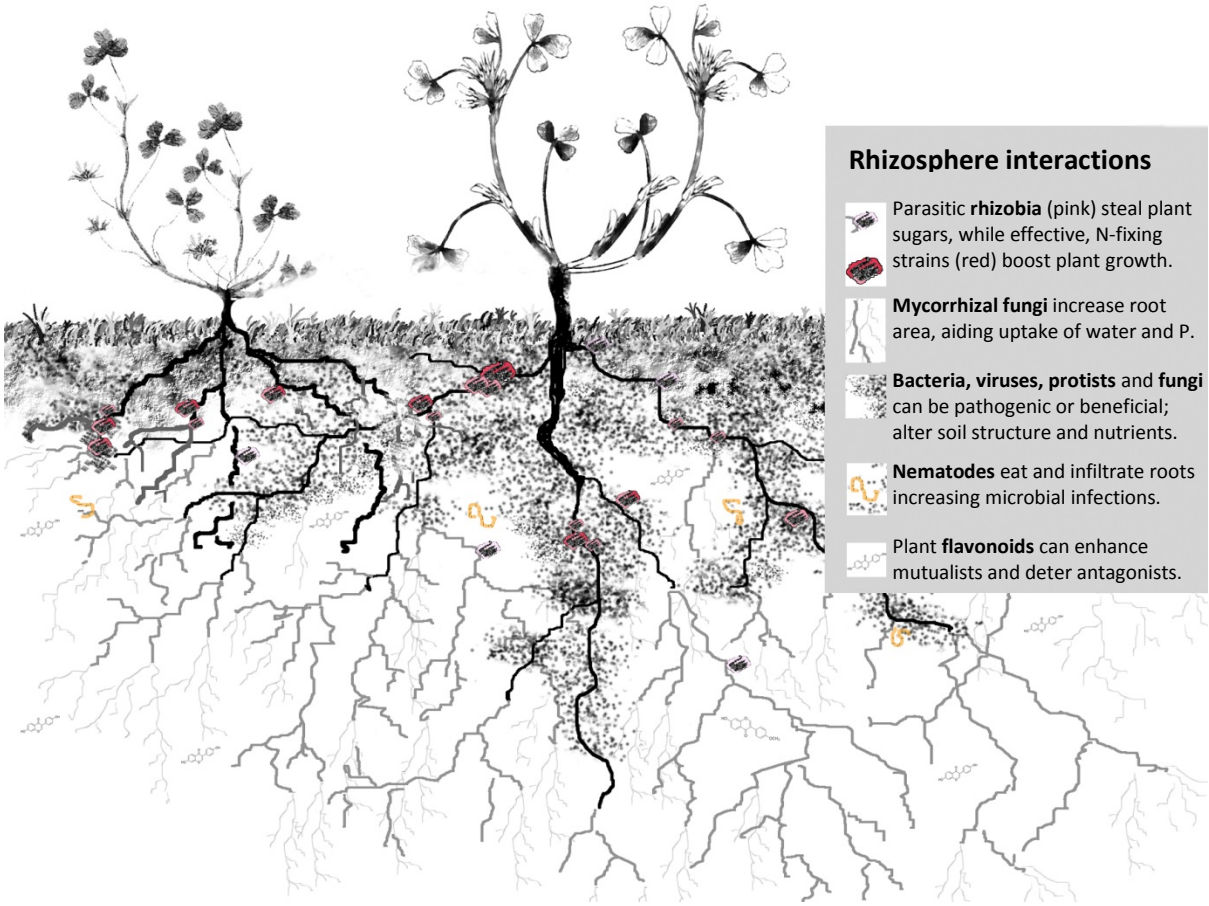


Figure 1.1. Most plant-microbe interactions take place in the "rhizosphere," the region closest to plant roots that is rich with antagonistic, mutualistic and saprophytic microbiota. These organisms have profound effects (both positive and negative) on plant performance and competitive ability. Many of the plant-microbe interactions in the rhizosphere are mediated by flavonoids, secondary metabolites produced by plants constitutively (independent of stimuli) and in response to specific microbial signatures.

Biogeographic differences in rhizosphere microbiomes have been revealed in two key areas that suggest these communities deserve greater attention in the context of invasions. First, there is evidence that some rhizosphere antagonists are absent in the introduced range of many plants species (Agrawal *et al.* 2005; Liu, Stiling & Pemberton 2007; Feng *et al.* 2009; Chun, van Kleunen & Dawson 2010; Hornoy *et al.* 2011), as plant-soil feedback experiments show that soil biota in a plant's native range are generally negative to its growth, whereas the soil biota in the non-native range tend to be positive or neutral (Bever *et al.* 1997, 2010; Willis, Memmott & Forrester 2000; Mitchell & Power 2003; Torchin *et al.* 2003; Levine *et al.* 2006; Inderjit & van der Putten 2010; Philippot *et al.* 2013; Gundale *et al.* 2014). Second, some plants fail to establish or perform poorly when the non-native range lacks their co-evolved symbiotic endophytes (Richardson *et al.* 2000a; Nuñez, Horton & Simberloff 2009; Dickie, Davis & Carswell 2012; Wandrag *et al.* 2013), and they do not benefit from the symbionts available in the non-native range (Traveset & Richardson 2011). Cumulatively, these differences suggest the novel rhizosphere communities plants encounter in their non-native range play key roles in plant performance, with important implications for post-naturalisation adaptation and altered competitive ability (Bossdorf *et al.* 2005; Yoder *et al.* 2010; Ellers *et al.* 2012).

1.3 Root-rhizosphere mutualisms

Plants derive enormous benefit from associating with rhizosphere mutualists, including increased access to water and nutrients (Selosse *et al.* 2006), buffering from abiotic stress (Abd-Alla *et al.* 2014), protection against soil pathogens and nematodes (Abe 2003), and increased competitive ability (van der Putten & Peters 1997; Sabais *et al.* 2012). Colonisation by some rhizosphere mutualists can even induce a plant's systemic resistance, making them less likely to be attacked by pathogens or nematodes (Pieterse *et al.* 2014). Because they have such a substantial impact on plant performance, rhizosphere mutualists can affect invasions at all stages—beginning with initial plant establishment or failure. For example, when mutualists are lost in the course of a plant being introduced to a new region (Traveset & Richardson 2011), a more mutualist-dependent host plant may be unable to establish (Richardson *et al.* 2000a) or may perform poorly (Wandrag *et al.* 2013) unless they can compensate (Ellers *et al.* 2012) or adapt (Seifert *et al.* 2009).

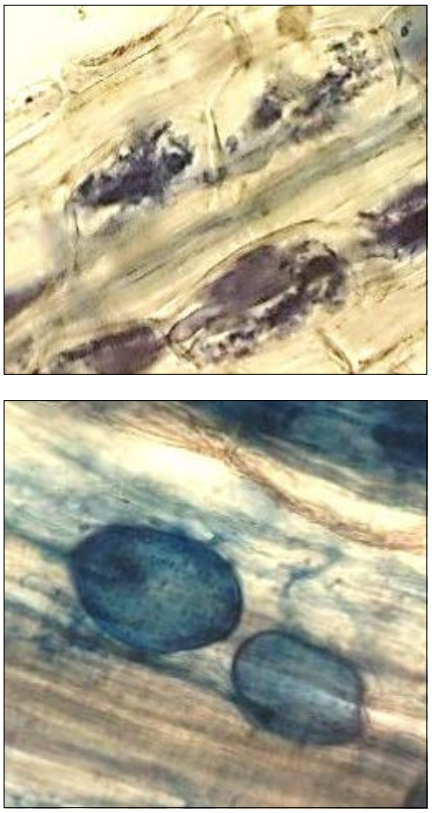


Figure 1.2. Arbuscules (top) and vesicles of arbuscular mycorrhizal fungi in *Trifolium* roots. (100x)

Although rhizosphere microbial mutualists are increasingly recognised as important players in plant performance and community dynamics (Schulz *et al.* 2006), their role in invasions has been understudied and challenging to untangle because (i) "failed" invasions are difficult to identify, so mutualist-related constraints can go undetected (Lockwood *et al.* 2007); (ii) the positive effects of escaping antagonists can cancel out the negative effects of losing mutualists (Callaway *et al.* 2011); (iii) naturalising plants may adapt to lost mutualists (Seifert *et al.* 2009); (iv) interactions with microbes are spatially and temporally dynamic (Thrall *et al.* 2007b), making patterns difficult to detect as communities change, as microbes from a plant's native range "catch up" with their hosts, and as pathogenic microbes in the new range accumulate (Diez *et al.* 2010; Hawkes, Douglas & Fitter 2010; Flory & Clay 2013); and (v) non-native plants may alter their new environments, e.g. by exuding allelopathic compounds (He *et al.* 2009) or altering nutrient regimes (Schweitzer *et al.* 2008)—thus the importance of a particular mutualist may also change over time. Importantly, little is known about what organisms compose soil microbiomes that associate with different plant communities—even in the most highly studied agricultural contexts.

Two of the most well-known and widely studied rhizosphere mutualists are mycorrhizal fungi (Figure 1.2) and nitrogen-fixing rhizobia bacteria (Figure 1.3). Both of these microbial endophytes provide substantial performance benefits to their plant hosts, both by increasing availability of nutrients but also serving as protection against environmental stress and pathogens (Schulz *et al.* 2006). Both form visible structures, making them easy to identify and therefore good candidates for studying altered mutualistic associations because it's possible to quantify the extent of their association with plant hosts.

1.3.1 Mycorrhizal fungi

Mycorrhizal fungi are the most common plant root symbionts, with at least 80% of land plants associating with one or more mycorrhizal species, and many associating with several taxa (Pringle *et al.* 2009). Most mycorrhizae are either arbuscular (those that penetrate root cells and form "tree-like" arbuscules) or ectomycorrhizal (those that infiltrate the root epidermis and/or cortex, but do not enter cells). Mycorrhizae colonisation can exponentially increase root surface area, acting like root hairs, thereby improving uptake of water and nutrients (Smith & Read 2010); in exchange, the plant provides photosynthesis-derived sugars to the fungus. Arbuscular mycorrhizal fungi (AMF) have been shown to be particularly important for phosphorus uptake, as less than 5% of soil phosphorus is bioavailable (Bulgarelli *et al.* 2013). Although mycorrhizae are considered somewhat generalist mutualists because they can form associations with various taxa and they are widespread globally (Pringle *et al.* 2009), the absence of certain mycorrhizal taxa can be a barrier to the establishment and spread of some plant species when they naturalise outside the native range. For example, *Pinus*

radiata (Pinaceae) was unable to spread outside of New Zealand plantations until a species of ectomycorrhizal fungi was introduced; after which the tree became widely naturalised throughout New Zealand's South Island in just a few decades (Richardson *et al.* 1994).

1.3.2 Nitrogen-fixing rhizobia

Nitrogen-fixing rhizobia are a taxa-specialised plant mutualist (Parker 1999; Rodríguez-Echeverría *et al.* 2012) mostly limited to associating with species in the Fabaceae (legume) family. Rhizobia colonise roots intercellularly, forming macroscopic nodules where they fix atmospheric dinitrogen into ammonia via the nitrogenase enzyme complex ($N_2 + 8H^+ + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$). This symbiosis provides the most limiting plant nutrient, nitrogen, directly to plant tissues and excess nitrogen is excreted into the soil, thereby boosting the growth of plants in the local community (Hirsch, Lum & Downie 2001). Rhizobia with high species-strain effectiveness are commonly "seeded" into agriculturally managed soils with their legume hosts, negating the need for synthetic fertilisers (Hirsch *et al.* 2001). Although some partners are generalists—one *Rhizobium* species, NGR234, can nodulate with at least 112 plant species—it is more common for plant-rhizobia symbioses to be species-, biovar- or strain-specific (Cooper 2004). Plant-microbe specificity arises from the complex chemical signals, mostly flavonoids, that are secreted into the rhizosphere by both partners (Hassan & Mathesius 2012); these signals are discussed in further detail below, in Section 1.4.

Figure 1.3. Nitrogen-fixing rhizobia nodules with characteristic red-purple colouring of laeghaemoglobin, an oxygen-binding protein, indicating active nitrogen-fixation. Roots shown are from *Trifolium striatum* colonised by *Rhizobium leguminosarum* biovar. *trifolii.*

1.3.3 Post-naturalisation mutualist interactions

While most studies of post-naturalisation adaptations have focussed on the role of enemies, it has been hypothesised that the early advantage of enemy release may attenuate with time as enemies "catch up" (Mitchell & Power 2003; Hawkes 2007a; Diez *et al.* 2010; Mitchell *et al.* 2010), so

rhizosphere mutualisms may become relatively more important as naturalisation progresses. The enormous performance benefit derived from hosting beneficial rhizosphere mutualists such as AMF and rhizobia suggests that when plants lose such mutualists in the course of naturalisation, they will either fail to naturalise, the "missed mutualists" hypothesis (Mitchell *et al.* 2006), or they will compensate via adaptive physiological traits, such as developing finer root architecture (Seifert *et al.* 2009), allocating more energy to belowground biomass, or producing different types or concentrations of root metabolites or soil exudates (Lankau 2012). Adaptations may also include shifts away from functional traits that stimulated or cultivated the association (Seifert *et al.* 2009), which may enable plants to divert resources to fitness (Lankau & Nodurft 2013), similar to EICA's reallocation predictions. Adaptations in response to lost or altered mutualists in the non-native range have not been given the same level of attention as antagonists, but studies suggest selection related to mutualists may be just as likely (Seifert *et al.* 2009; Porter, Stanton & Rice 2011; Lankau & Nodurft 2013). For example, adaptation can arise from reduced mutualist availability. *Hypericum perforatum* (Hypericaceae), that disassociated from highly compatible co-evolved AMF developed thinner roots and lower shoot-root (S:R) ratios (Seifert *et al.* 2009), two morphological characteristics associated with non-mycorrhizal plants (Tawaraya 2003). When plants from these non-native populations were re-exposed to AMF in glasshouse experiments, they incurred significantly less growth benefit from the association than native conspecifics, suggesting the shifts in root traits were fixed adaptations resulting from separation from the mutualist.

Alternatively, plants may encounter novel beneficial endophytes (or strains) in the non-native range and may adapt to associate more effectively with them. For example, although AMF are cosmopolitan and can be found in most environments (Richardson *et al.* 2000a), individual AMF populations are dispersal limited (Pringle *et al.* 2009) and many taxa are correlated with particular plant communities (Martinez-Garcia *et al.* 2015), so plants that naturalise outside their native range are likely to interact with different AMF species compared with those with which they interacted in their native range. Although many AMF presumably lack a high degree of taxon specificity (Mar Vázquez *et al.* 2000), the degree of nutrient acquisition and growth benefit to the plant will certainly differ among strains as a result of environmental conditions, genotypes and the history of association with a particular microbe (Mallory-Smith *et al.* 1990; Wright, Scholes & Read 1998b; Eason *et al.* 2001; Seifert *et al.* 2009; Callaway *et al.* 2011; Nuñez & Dickie 2013; Bunn *et al.* 2015). For example, plant-soil feedback experiments show that AMF strains cultured from a plant of the same species are more beneficial to that plant's growth (Klironomos 2002). Similarly, in California grasslands, non-native *Medicago polymorpha* (Fabaceae) exhibit site-specific adaptations that result in greater investment to the nitrogen-fixing rhizobia strains that are locally available (Porter *et al.* 2011). Evidence for rapid adaptation related to mutualist association is documented among native

populations of *Pilea pumila* (Urticaceae) in response to invasion by the non-mycorrhizal plant *Alliaria petiolata* (Brassicaceae), which inhibits AMF. In this system, *P. pumila* with long histories of *A. petiolata* invasion have adapted to sustain a more diverse mycorrhizal community (Lankau & Nodurft 2013), presumably so that they can take advantage of AMF that are resistant to *A. petiolata* exudates

An added layer of complexity in post-naturalisation differences related to mutualist associations is that although colonisation by a rhizosphere mutualist is typically assumed to indicate a beneficial interaction (Paszkowski 2006), both mycorrhizal fungi and rhizobia bacteria can be parasitic (Greenwood 1964; Harrison, Young & Jones 1989; Johnson, Graham & Smith 1997; Klironomos 2003; Denison & Kiers 2004a; b; Schwartz *et al.* 2006; Desprez-Loustau *et al.* 2007; Drew *et al.* 2011). The prevalence of parasitism is largely unknown even in agriculturally managed fields, let alone in natural communities or invaded systems (Pryor *et al.* 2004; Denison & Kiers 2004b). Even in the rhizobia-legume symbiosis, which has been maintained in agricultural settings for centuries and manipulated genetically for decades, the mechanistic interactions between microbe and host remain poorly understood, and genetic similarity does not always reliably predict symbiotic effectiveness (Yates *et al.* 2008). For example, two OTUs with greater than 97% similarity (based on ribosomal RNA) can have genes driving substantially different ecological functions (Rout & Callaway 2012). Thus, rhizobia that are considered "compatible" or even seen as genetically identical can be highly effective nitrogen fixers or so parasitic that they kill the host plant (Denison & Kiers 2004a). Differentiating mutualistic versus parasitic strains by genetic testing is hampered because most root nodules are heterogeneous (containing a mixture of rhizobial strains), with some fixing nitrogen and some only taking photosynthate from the plant host (Denison & Kiers 2004a). The unknown range of costs and benefits in plant-microbe interactions complicates efforts to understand how these relationships may differ during plant naturalisation events. Thus, empirical field and glasshouse studies are needed to reveal the facultative role of microbial strains in each range and thus how plants from different provenances may interact differently with microbiota in each range.

In summary, while rhizosphere mutualist absence or the presence of non-beneficial/parasitic strains can contribute to naturalisation success or failure, such altered mutualisms can alternatively stimulate post-naturalisation shifts in physiology that may result in physiological compensations or adaptations.

1.3.4 Root flavonoids: Agents of defence, mediators of mutualisms

The interactions between plants and rhizosphere microbes are coordinated by signals composed of amino acids, polypeptides, polysaccharides, fatty acids, and complex secondary metabolites. Of these, flavonoids, a diverse class of polyphenolic compounds, are considered some of the most important mediators of plant-microbe interactions—serving as both the hypersensitive agents at the

front-line of plant defence (Sabudak & Guler 2009) and the hormone-like coordinators of colonisation by plant growth-promoting endophytes (Dakora & Phillips 1996; Bais *et al.* 2004; Andersen & Markham 2006; Cooper 2007). Flavonoids are produced in plant tissues both constitutively (independent of stimuli) and in response to specific chemical cues (Andersen & Markham 2006; Tahara 2007; Sisa *et al.* 2010). In the rhizosphere, root flavonoid functions include stress protection, herbivore/pathogen resistance (Sabudak & Guler 2009), allelopathy, (Sisa *et al.* 2010), and chelation of soil nutrients (Hassan & Mathesius 2012). Although substantial levels of flavonoids can be found in plant tissues even under sterile conditions (Dakora & Phillips 1996), when plants are exposed to certain biota, gene transcription in the phenylpropanoid pathways is activated and concentrations of some flavonoid types, particularly isoflavonoids, can peak within minutes (Stafford 1997). For example, the chemical signature of an attacking soil pathogen can trigger the production of specific combinations and concentrations of defence flavonoids that can concentrate in root tissues and also be exuded into the rhizosphere to inhibit further attack (Stafford 1997; Bais *et al.* 2006; Hassan & Mathesius 2012; Tuominen 2013).

Flavonoids also act as stimulants—biochanin A and formononetin induce and direct root colonisation by AMF (Nair, Safir & Siqueira 1991; Wright, Scholes & Read 1998a; Osmond 1999) in concentrations of as low as 5 parts per million (ppm) (Nair *et al.* 1991) and the isoflavonoid daidzein initiates nodulation with nitrogen-fixing rhizobia (Howieson et al. 2005; Chatel & Greenwood 1973; Miller et al. 2007). The mutualism with rhizobia is one of the most specific and well described chemical exchanges in the rhizosphere. Molecular signalling begins when the plant releases flavonoids from seeds or roots to stimulate rhizobial chemotaxis, causing bacterial cells to concentrate near the root (Cooper 2004). Next, the plant releases flavones that stimulate rhizobial nod factors in the prokaryote (Peck et al. 2006) and, if the nod factors match the plant host (Dakora & Phillips 1996), the plant secretes yet another set of flavonoids that alter the formation of root cell surface polysaccharides to initiate colonization (Cooper 2004, 2007). This entire exchange of chemicals is so specific that if a rhizobial strain is not the right match, plants may release flavonoids in a different combination or concentration to inhibit colonisation (Dakora & Phillips 1996).

1.3.5 Post-naturalisation biochemical shifts

Plant synthesis of secondary metabolites is often specific to stimuli (Jung *et al.* 2000; Andersen & Markham 2006; Tahara 2007; Sisa *et al.* 2010), so the composition and abundance of these metabolites will likely be subject to selection when a non-native plant is introduced to a new rhizosphere community and different environmental conditions (Bever 2003; Wardle *et al.* 2004; Mitchell *et al.* 2006; Kardol *et al.* 2007; van der Putten *et al.* 2007; Revilla *et al.* 2012; Rout & Callaway 2012; Bardgett & van der Putten 2014). Biochemical shifts have been reported in response

to altered UV radiation (Hoffman 2000) and in response to changes in herbivory. EICA predicts that if a plant escapes a native-range pathogen in the non-native range, it may decrease the synthesis of any specialised defence compounds that protected it from that pathogen (Blossey & Nötzold 1995; Reinhart & Callaway 2006; van der Putten *et al.* 2007; Sun, Müller-Schärer & Schaffner 2014). Mutualism-related trade-offs have not been studied with the same rigor as enemies, but when plants lose co-evolved enemies they may also down-regulate flavonoids that stimulated or enhanced a rhizosphere mutualism (Kiers *et al.* 2010), even ceasing production of certain types of compounds (Keller & Taylor 2008) if this provides a fitness advantage.

Selection pressure related to both antagonists and mutualists may be particularly strong when flavonoids are both produced constitutively (regardless of stimuli) and are energetically costly (Hartmann 2007; Foyer, Noctor & van Emden 2007; Beaton *et al.* 2011; Bekaert *et al.* 2012) such as a flavonoid exudate that is produced regularly to help a particular endophyte find the plant host (Cooper 2007). If this "call" goes unanswered (i.e. the mutualist is absent and there is no advantage to producing the exudate) synthesis of the flavonoid represents a cost without benefit. Numerous studies have found that plants shift foliar chemical profiles in the non-native range (Herms & Mattson 1992; Peñuelas *et al.* 2010; Felker-Quinn *et al.* 2013) and analogous trends are now being uncovered in belowground systems (Seifert *et al.* 2009; Bever 2015).

1.4 New Zealand *Trifolium* as a model system

As a model system for investigating post-naturalisation differences in plant traits, I use non-agricultural species in the genus *Trifolium* (Fabaceae), the "true clovers" (Zohary & Heller 1984) that are native to Europe and have naturalised in New Zealand (Gravuer 2004). In New Zealand, agricultural *Trifolium* species have a long naturalisation history and are reseeded regularly to encourage vigorous genotypes (Castle 2000) as intense breeding programmes exist throughout the country (Wratt & Smith 2013). As a result of more than a century of *Trifolium* seed import, many non-agricultural species have been accidentally introduced as seed contaminants (Gravuer *et al.* 2008), and 16 of the 54 introduced species have naturalised (Gravuer 2004). Although even the accidentally introduced clover*s* are not considered problematic in New Zealand, the genus serves as a strong model for theoretical work in invasion ecology because it (i) is well-studied, as a result of its importance to agriculture; (ii) naturalises globally in a variety of habitats—including roadsides, pastures, woodlands and alpine areas (Cronk & Fuller 2001; Parker & Gilbert 2007), making it possible to integrate a variety of environment types; (iii) forms partnerships with well-studied and easily quantifiable rhizosphere mutualists; (iv) produces copious secondary metabolites relevant to both mutualism and defence (Sabudak & Guler 2009); (v) can adapt rapidly in response to altered conditions such as UV, frost, grazing, etc. (Williams, Plummer & Phung 1982; Caradus 1994; Olsen,

Hsu & Small 2008; Hofmann & Jahufer 2011); and (vi) co-occurs throughout New Zealand with agricultural conspecifics, grasses and forbs, suggesting species face intense competition and will be subjected to the selection pressures predicted by the EICA hypothesis. Another benefit of using the New Zealand *Trifolium* as a model system is that there are no native congeners and thus no native clover rhizobia, so the islands provide a more controlled and isolated environment for studying invasions. Although New Zealand has its own suite of native legumes (including the well-known genera *Sophora, Carmichaelia,* and *Clianthus)*, these are primarily trees and shrubs that do not co-occur in the same habitats as *Trifolium,* which most commonly colonise roadsides, grasslands, and ruderal areas. Indeed, in this study, the only legumes found at the study sites were non-native legumes, notably *Trifolium* and *Medicago.*

Like many legumes, *Trifolium* present an interesting dichotomy—their species-strain specificity with rhizobia and their tendency to host multiple soil symbionts that rely on each other's presence suggests they should be mutualist limited and thus poor invaders—indeed, 38 of the 54 species of *Trifolium* that have been accidentally introduced to New Zealand failed to naturalise (Gravuer *et al.* 2008). Yet, many non-agricultural *Trifolium* species are globally successful naturalisers (Parker *et al.* 2006) and two agricultural species, *T. repens* and *T. dubium,* are listed in the Global Invasive Species Database (Invasive Species Specialist Group 2015). A study by Gravuer et al. (2008) found that *Trifolium* that were accidentally introduced to New Zealand and that had succeeded in naturalising were typically those characterised by having a large native range, wide tolerance to diverse conditions, a native range that matched the climate in New Zealand and that had extensive opportunity to be transported as seed contaminants. However, even the strongest model proposed by these authors only explained about 56% of the variation. This led me to predict that the dichotomy of mutualist-dependence but successful invader might be explained by post-naturalisation trait shifts specifically related to differences in rhizosphere microbial mutualists between the native and non-native ranges.

I chose seven *Trifolium* species with different distributions in New Zealand, but that have been naturalised on the islands for 84–160 years (Table 1.1), suggesting sufficient time for adaptation to occur (Atwood & Meyerson 2011). Analyses were limited to the non-agricultural species because pasture clovers are highly manipulated genetically for enhanced rhizobia mutualism and resistance to pests, whereas I wished for this work to serve as a theoretical platform that informs on the prevalence and magnitude of trait shifts that may occur when plants naturalise outside their native ranges. Rapid adaptation and trait shifting in response to both environmental differences and community interactions has already been shown for *T. repens* (Whitman 1973; Hofmann 2000; Hofmann & Jahufer 2011), and *T. glomeratum* in Australia (Buswell *et al.* 2011). A criticism of previous tests of EICA has been that differences between native and non-native provenances are

often interpreted as adaptation related to selective pressure, without taking into account the role of stochastic phenotypic evolution (Keller & Taylor 2008). A benefit of the approach used in this thesis—multiple species from the same genus with a range of naturalisation dates—is that these populations will have been subjected to various stochastic factors, but similar biotic and abiotic factors. This enables a more direct investigation of post-naturalisation differences that have been shaped by the conditions of the non-native range and minimises the role of chance or founder effects in the results.

Using species that have a range of geographic distributions in their non-native range allows one to make inferences about how post-naturalisation performance differences may correlate with naturalisation success Thus, I specifically included a species that is widely naturalised (*T. arvense*) and three that are naturalised but not common in New Zealand (*T. micranthum, T. ornithopodioides, T. tomentosum)* (Table 1.1*,* Figure 1.2). For the seven species, there is no correlation between New Zealand naturalisation date and countrywide geographic extent (Pearson's correlation = -0.34, P = 0.45) (Figure 1.5) eliminating the potentially confounding factor of residence time (Richardson *et al.* 2000b; Gravuer 2004). I also looked for correlations between inter-provenance performance differences and regional distribution because *T. glomeratum, T. arvense* and *T. tomentosum* are locally abundant in the study area in New Zealand, whereas the other four species are uncommon.

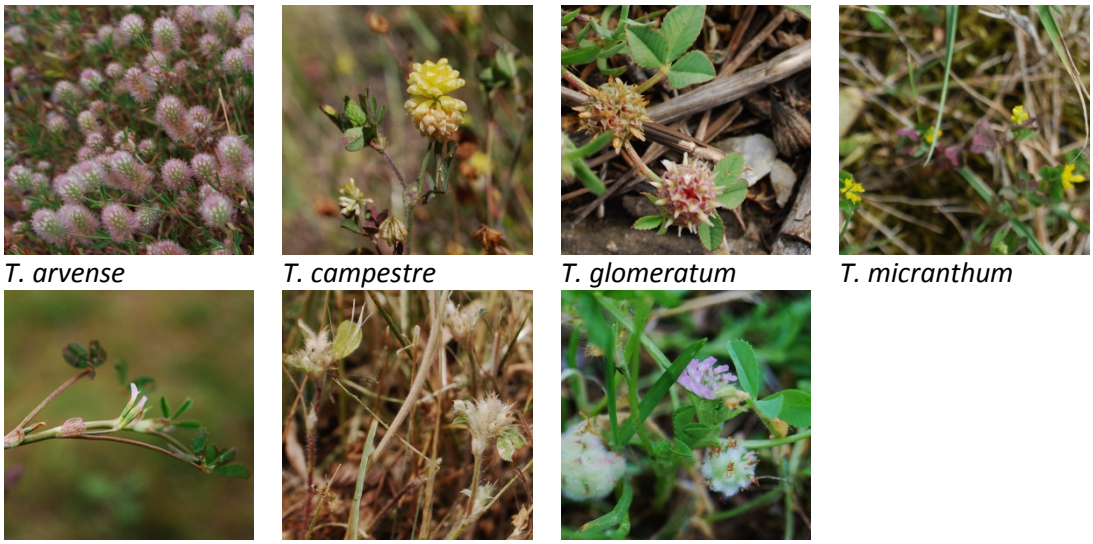


Figure 1.4. The seven species of non-agricultural, annual *Trifolium* used in this thesis.

1.4.1 Antagonists of *Trifolium* in New Zealand

Trifolium are host to a large number of above- and belowground antagonistic biota in both their native and non-native ranges, including pathogenic soil bacteria and fungi, root nematodes and herbivorous invertebrates (Zohary & Heller 1984; Manolitz 1985; Mercer & Miller 1997; Hayden & Parker 2002; Wang, Ridsdill-Smith & Ghisalberti 2005). In New Zealand damaging biota on agricultural *Trifolium* include the clover root weevil, grass grub, Porina and white-fringed weevil and clover casebearer, and stem, root-knot, root-rot and cyst nematodes and several genera of root-invading fungi (Skipp & Christensen 1983; Skipp & Watson 1987; Wratt & Smith 2013). Although the extent of antagonist biota has not been quantified for the accidentally introduced species, congeners often share pests (Gilbert & Parker 2010), so the high incidence of antagonists found on the agricultural *Trifolium* in New Zealand suggests that the non-agricultural species are subjected to similar levels of attack. Thus, given the numerous pests and pathogens of *Trifolium* in New Zealand, and assertions that *Trifolium* naturalise and persist in a variety of environments regardless of the composition of pests (Wratt & Smith 2013), in this system we might expect that the effect of losing optimal strains of rhizosphere mutualists may have an even greater effect than escape from co-evolved antagonists—thus special attention is given to the role of mutualists in this thesis.

1.4.2 Rhizosphere mutualists of *Trifolium* in New Zealand

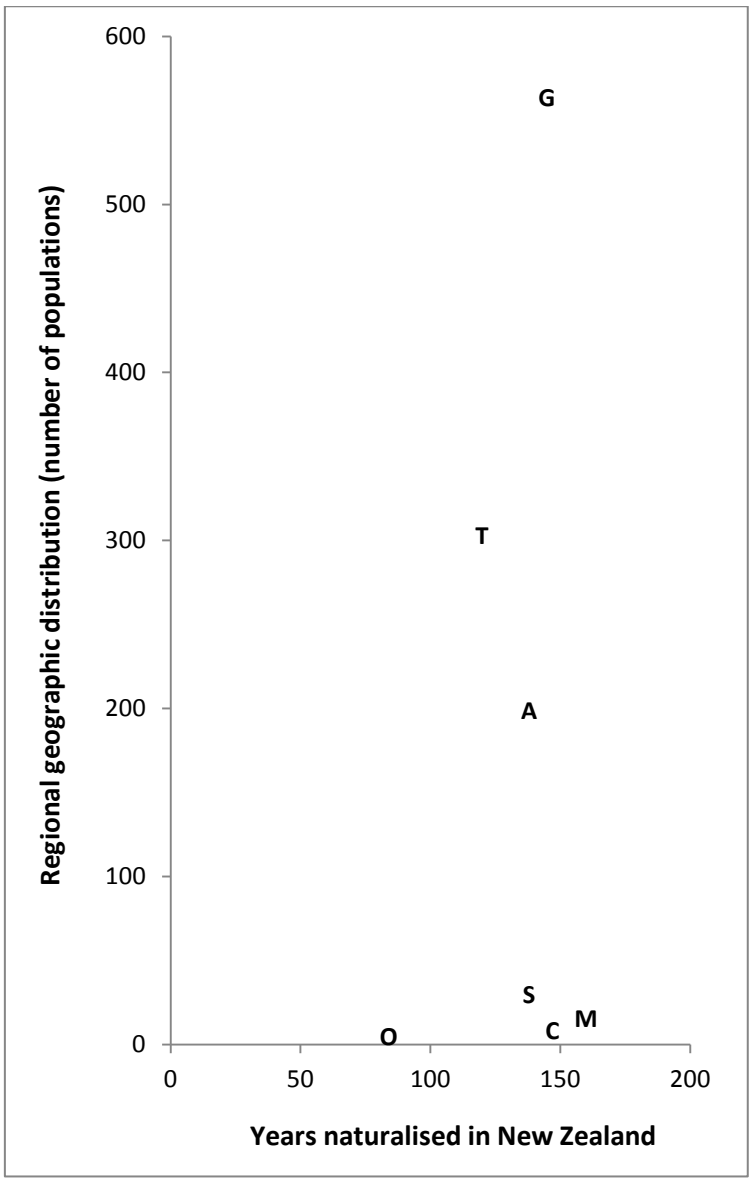
Arbuscular mycorrhizal fungi (AMF) are widespread in New Zealand—several genera are native and many species and strains have been introduced for agriculture (Scott 1975), although their extent, richness, and specificity remains unexplored (but see Eason *et al.* 2001; McGinn 2015). *Trifolium* species, including two of the most common pastoral species in New Zealand, *T. repens* and *T. subterraneum,* associate with many species of AMF in both their native and introduced ranges (Nair *et al.* 1991; Wright *et al.* 1998b; Osmond 1999). The extent of AMF colonisation of non-agricultural species is largely unknown, however one would expect high levels of AMF (or root-trait compensation) in New Zealand because these soils are phosphorus poor (Sarathchandra *et al.* 1984; Boswell *et al.* 2003). Indeed, pastoral clover systems often provide regular phosphorus input to maintain clover populations (Haynes & Francis 1990), suggesting that these clovers are not deriving sufficient benefit from AMF in these soils. In addition, the rhizobia mutualism requires high levels of phosphorus for successful nitrogen fixation (Abd-Alla *et al.* 2014), so a positive correlation between AMF and rhizobia colonisation is predicted in all soils (Scott 1975; Xie *et al.* 1995).

Although there are native legumes and native rhizobia in New Zealand (Weir 2006), *Trifolium* spp. only associate with one rhizobia biovar, *Rhizobium leguminosarum* bv. *trifolii,* and thus abundant rhizobia strains have been introduced for the agriculturally important species (Lowther & Kerr 2011). Although there is some strain sharing among *Trifolium* species—e.g. *T. arvense* and *T. glomeratum*

belong to the same rhizobia "effectiveness group"—each species has its own suite of optimal rhizobia strains (Howieson et al. 2005; Greenwood 1964; Chatel & Greenwood 1973; Miller et al. 2007). Experiments of strain effectiveness (Howieson *et al.* 2005; Yates *et al.* 2005) suggest that non-agricultural clovers and particularly annuals of European origin are unlikely to benefit much from colonisation by the agricultural strains (even though several species, including *T. arvense, T. glomeratum and T. striatum* can nodulate with agricultural rhizobia) (Denton *et al.* 2003; Drew *et al.* 2011). Although there are many strains of agricultural rhizobia in New Zealand (Nangul *et al.* 2013), and richness of strains colonising *Trifolium* may even be comparable to levels in the native range (McGinn *et al.* 2016), nodulation of agricultural rhizobia has been historically low on non-agricultural annual *Trifolium*, probably because plants must be colonised from free-living populations of rhizobia in the soil, and non-agricultural clovers were most likely introduced to New Zealand as seed contaminants (Gravuer 2004). In addition, between plant generations the rhizobia for annual *Trifolium* species have to compete for space and nutrients in the soil matrix with the naturalised agricultural strains ((Greenwood 1964) known to be ubiquitous in New Zealand soils—even far from pasturelands and in the absence of their plant hosts. For example, the eastern European annual *T. ambiguum* has failed to naturalise outside its native range, including in New Zealand, reportedly because of the lack of its specialist strain of rhizobia (Beauregard *et al.* 2003). Lack of sufficient compatible rhizobia could explain the naturalisation failure of 38 clovers that have been introduced to New Zealand since the late 1800s (Gravuer 2004). From the perspective of study design, the rhizobia specificity of each species is an advantage because each *Trifolium* species serves as an independent replicate with its own response to the suite of rhizobia in the non-native range; whereas previous tests that looked at the role of rhizobia mutualist in invasions focussed solely on promiscuous (i.e. less taxa-specialised) plant-rhizobia interactions (Wei *et al.* 2009; Rodríguez-Echeverría *et al.* 2012; Crisóstomo, Rodríguez-Echeverría & Freitas 2013; Wandrag *et al.* 2013).

Species letter codes: A = T. arvense, C = T. campestre; G = T. glomeratum; M = T. micranthum; O= T. ornithopodioides; S = T. striatum; T = T. tomentosum

Figure 1.5. Naturalisation date plotted as a factor of extent of distribution (total number of 30 km x 40 km NZMS260 grid cells occupied by at least one population) in New Zealand for the seven species of accidentally introduced *Trifolium* used in this study (Data from Gravuer 2004).



Species letter codes: A = T. arvense, C = T. campestre; G = T. glomeratum; M = T. micranthum; O= T. ornithopodioides; S = T. striatum; T = T. tomentosum

Figure 1.6. Naturalisation date plotted as a factor of extent of regional distribution (total number of populations in the study area in the non-native range, Banks Peninsula, New Zealand) for the seven species of accidentally introduced *Trifolium* used in this study (Data from a 1983-1988 vegetation survey of Banks Peninsula and local environs, (Wiser, Bellingham & Burrows 2001).

Table 1.1. Summary information for the seven species of *Trifolium* used in this study.

Species	Year naturalised in New Zealand*	Distribution		
		Banks Peninsula, NZ populations †	Total New Zealand NZMS grids ǂ	Native range area inhabited (x 10^{12} km^2¶
T. arvense L.	1880	199	83	26.6
T. campestre Schreb.	1867	9	46	20.1
T. glomeratum L.	1870	564	44	7.7
T. micranthum Viv.	1854	16	23	10.0
T. ornithopodioides L.	1930	5	26	5.4
T. striatum L.	1878	301	44	10.1
T. tomentosum L.	1948	30	21	11.4

* Data from Gravuer 2004
† Single-population records based on a 1983-1988 vegetation survey of Banks Peninsula and local environs; nvs.landcareresearch.co.nz; Wiser et al. 2001
ǂ Number of 10 x 10 km NZMS260 grids occupied by at least one population; Gravuer 2004
¶ Area estimate (x 10^{12} km^2); Gravuer 2004

1.5 Thesis objectives and outline

In this thesis, I use a globally naturalised genus, *Trifolium,* as a model taxon to look for evidence of post-naturalisation adaptations. Expanding upon the framework of previous tests of the EICA hypothesis, my investigations compare the performance of plants from native and non-native provenances when grown in rhizosphere soil cultivated *in situ* by conspecifics in each range. Previous plant-invasion studies have not addressed differences in performance nor tested for increased competitive ability while incorporating rhizosphere microbial communities in each range. I aimed to answer two fundamental questions:

- Do non-native plants exhibit performance or trait differences that may be evidence for post-naturalisation adaptation?

- Are there trends in post-naturalisation differences that correlate with invader success?

To address these questions, I conducted three empirical tests, each one aiming to fill a knowledge gap in studies that investigate post-naturalisation adaptation: the role of mutualists, the production of root flavonoids, and differences in competitive ability. In each test, I grew plants sourced from provenances in the native (Spain and the UK) and non-native (New Zealand) ranges in glasshouse pots inoculated with rhizosphere soil cultivated by conspecifics *in situ* in each provenance. While

most tests looking for post-naturalisation adaptations usually focus on a single, problematic invader and compare the performance of one or more populations from each range, in this thesis I took a coarse-scale, theory-based approach, using species as the form of replication to look for consistent evidence of post-naturalisation adaptation among members of a globally naturalised genus.

In Chapter 2, I ask whether differences between the native and non-native ranges have resulted in plants from the non-native range having lower levels of colonisation and growth benefit from two common root mutualists—nitrogen-fixing rhizobia and arbuscular mycorrhizal fungi. I also look for evidence of physiological compensation in plant traits (e.g. lower shoot-root ratios).

In Chapter 3, I ask whether plants in the non-native range, having lost co-evolved rhizosphere microbial communities, have decreased root flavonoid concentration and richness. I test the effect of rhizosphere community by using sterilised and unsterilised rhizosphere soil treatments in both the native and non-native ranges. I also look for evidence of energetic trade-offs that would support the optimal-defence predictions of the EICA hypothesis (e.g. inverse correlations between growth rates and flavonoid production).

In Chapter 4, I use three of the most widely naturalised clovers in New Zealand to test the EICA hypothesis that plants from non-native provenances have adapted increased competitive ability and that this increased performance is directly correlated with their naturalisation success (i.e. geographic extent in New Zealand). Surprisingly, post-naturalisation competitive ability has been understudied in EICA investigations (Felker-Quinn *et al.* 2013). Most EICA tests use single-plant growth as a surrogate for competitive ability, or pair plants with a common heterospecific (but see Bossdorf *et al.* 2004). Although competition tests with heterospecifics are important to gauge how plants interact with the plant communities in the non-native range, these tests are confounded by species-specific interactions and cannot provide a measure of post-naturalisation change—the core prediction of this evolution-focused hypothesis. A recent meta-analysis of EICA showed that while post-naturalisation differences in performance are common, the few experiments that have performed direct tests of competition showed no support for a concurrent increase in competitive ability (Felker-Quinn *et al.* 2013). In this analysis, I use intra-specific tests of competition between plants sourced from seed in each range and grown together in rhizosphere soil cultivated by conspecifics from each range. This method not only enables detection of performance and competitive differences between plants from native and non-native seed provenances, but makes these comparisons in the context of the rhizosphere microbiota from each range. This is the first known multi-species EICA test that incorporates rhizosphere microbial communities and intra-specific measures of competition.

Chapters were written as self-contained research papers and thus there is some overlap and repetition among the data chapters (Chapters 2-4). Chapter 5 (General Discussion) functions as a synthesis of the thesis and elucidates the potential implications of my findings.

Chapter 2

Rhizosphere mutualist association lower among non-native *Trifolium*

2.1 Abstract

The success of many invasive plants has been attributed to their escape from population-controlling antagonistic biota in the native range; however many plants concurrently lose the benefits associated with native-range mutualists. The absence of these mutualists can be a barrier to naturalisation, yet many plants that form highly specialised mutualisms with dispersal-limited biota are still successful invaders. One explanation is that some invaders adapt to reduced mutualist availability post-naturalisation, by reallocating investments away from maintaining los mutualisms and toward other fitness traits. This study investigated whether plants from non-native provenances show evidence of adaptation to lost mutualists, and predicted that divergence would be correlated with the plant species' naturalisation success (geographic spread). A test was conducted using seven species of *Trifolium*—a globally naturalised genus that hosts both nitrogen-fixing rhizobia and arbuscular mycorrhizal fungi (AMF). Plants from native (Spain and the UK) and non-native (New Zealand) provenances were grown in glasshouse pots inoculated with rhizosphere soil cultivated by conspecifics from each provenance. Inter-provenance comparisons were made on mutualist colonisation levels and the benefit of association (growth rate increase as a factor of colonisation). In all soils, rhizobia nodulation was generally lower among plants from non-native provenances compared to native conspecifics and the growth benefit from rhizobia association was also lower. AMF colonisation varied with one significant inter-provenance difference in the direction predicted, and the growth benefit from AMF was significantly lower among non-native plants. However, there was no evidence for correlations between the inter-provenance differences in mutualist association and species' naturalisation success in New Zealand. Nor was there evidence for physiological compensation for the loss in root mutualist associations, as shoot-root ratios were similar among all plants in all soils, with the exception of UK soil, which showed a significant inter-provenance difference in the opposite direction predicted. Thus, for *Trifolium* in New Zealand, association and benefit with rhizosphere mutualists are reduced, but the loss does not appear to be responsible for the slower growth rates among non-native plants, nor does mutualist loss appear to stimulate differences in shoot-root allocation, nor does it appear to be a factor in the naturalisation success of these seven species.

2.2 Introduction

Plant-microbe interactions in the rhizosphere play a pivotal role in plant performance and the structuring of plant communities (Bardgett & van der Putten 2014) and therefore are key players during plant naturalisation. Plants introduced to new locations leave behind much of the microbiota of their native range, which potentially includes population-controlling pests, pathogens and herbivores (Agrawal *et al.* 2005; Liu *et al.* 2007; Feng *et al.* 2009; Chun *et al.* 2010; Hornoy *et al.* 2011), but also mutualistic rhizosphere microbiota that provide limiting resources, enhance plant growth and fecundity (Richardson *et al.* 2000a; Reinhart & Callaway 2006), buffer plants against abiotic stress (Abd-Alla *et al.* 2014) and protect against attack from nematodes (Abe 2003). Thus, while separation from antagonistic rhizosphere biota can facilitate invasions (Joshi & Vrieling 2005; Liu & Stiling 2006), the absence of certain rhizosphere mutualists can be a barrier (Parker, Burkepile & Hay 2006a; Rodríguez-Echeverría & Crisóstomo 2009) or at least a hindrance to plant performance (Wandrag *et al.* 2013).

Some invasive taxa, such as legumes (Fabaceae), host multiple specialised rhizosphere mutualists that rely on each other's presence (Stafford 1997), thus, legumes have been identified as one group where loss of soil mutualists could potentially impact their ability to establish and spread in new regions (Parker 2001; Rodríguez-Echeverría 2010; Rodríguez-Echeverría *et al.* 2012; Birnbaum *et al.* 2014). For example, *Trifolium* (true clovers), associate only with one biovar of nitrogen-fixing rhizobia, *Rhizobium leguminosarum* bv. *trifolii* (Howieson *et al.* 2005) and they also frequently form a synergistic, "tripartite" association with arbuscular mycorrhizal fungi (AMF) (Crush 1982; Bethlenfalvay, Newton & Regional 1991), an endophyte that exponentially increases a plant's root system, increasing plant access to water and soil nutrients, particularly phosphorus (Smith & Read 2010). Because nitrogen fixation requires substantial phosphorus input, in phosphorus-limited soils, rhizobia colonisation can fail unless the plant is colonised by AMF (Crush 1974); when both mutualists are present, nitrogen fixation increases substantially (Bethlenfalvay *et al.* 1991; Sprent & James 2007; Abd-Alla *et al.* 2014).

Despite these observed tripartite dependencies with microbial mutualists that are geographically constrained and taxon-specific, legumes(Fabaceae) are among the most widely naturalised plants (e.g. *Trifolium* spp.*, Robinia pseudoacacia, Cytisus scoparius, Ulex* spp., *Medicago* spp., *Acacia* spp.) (ISSG of the IUCN SSC 2015). Legume success may thus be attributed to one or more of the following explanations: (i) some rhizosphere mutualists are generalists and plants may encounter suitable taxa outside the native range; (ii) the benefit of escaping natural enemies outweighs the loss of a mutualist (e.g. *Robinia pseudoacacia* is separated from co-evolved AMF outside its native range, yet the release from pathogens outweighs the loss (Callaway *et al.* 2011)); or (iii) mutualists can be co-

introduced (e.g. legumes are economically important so their introduction to new regions is often accompanied by purposeful co-introduction of compatible rhizobia (Lowther & Kerr 2011)).

A fourth explanation that has not yet been widely tested is that non-native plants adapt to decreased availability of compatible soil mutualists (Seifert et al. 2009; Reinhart et al. 2010; Porter et al. 2011; Baynes et al. 2012) by diverting energy away from maintaining lost mutualists and either compensate physiologically or reallocate energy to other fitness characters. (Tawaraya 2003). In systems where this fourth explanation holds, over time we would predict non-native populations that have lost specialised rhizosphere mutualists to diverge from native populations—associating less with the mutualist when available and possibly developing physiological compensations to deal with this loss. For example, low shoot-root ratios are believed to be indicative of biomass compensation related to reduced belowground mutualist associations (Seifert *et al.* 2009); whereas higher shoot-root ratios indicate a strong benefit of belowground mutualists (Karst *et al.* 2008; Andonian & Hierro 2011), presumably because plants acquire nutrients at an elevated rate and invest more energy into photosynthetic structures, producing more photosynthate with less root biomass (Johnson *et al.* 1997; Agren & Franklin 2003). For example, Seifert *et al.* (2009) found that *Hypericum perforatum* (Hypericaceae) that have not been exposed to AMF for several generations have shown reduced association with AMF and incur significantly less growth benefit from AMF inoculation than native conspecifics. Moreover, these plants had finer root architecture and lower shoot-root ratios (Seifert et al. 2009), characters typically associated with non-mycorrhizal plants.

In this study, we test the prediction that plants adapt to associate less with rhizosphere mutualists in their non-native range. We use seven non-agricultural *Trifolium* species native to Europe that have widely naturalised in New Zealand. This system is a good candidate for investigating post-naturalisation adaptations related to lost rhizosphere mutualists because New Zealand soils are generally phosphorus- and nitrogen-poor and, although agricultural rhizobia strains have been introduced to New Zealand*,* surveys of their effectiveness (Howieson *et al.* 2005; Yates *et al.* 2005; Lowther & Kerr 2011) suggest that non-agricultural annual clovers of European origin (Denton *et al.* 2003; Drew *et al.* 2011) do not benefit greatly from them. Native and introduced agricultural strains of AMF are also widespread in New Zealand (Scott 1975; Eason *et al.* 2001), however the AMF common to New Zealand pastures (the likely entry point for these species (Gravuer 2004)) have been reported to be inefficient (Powell 1979) and the growth benefit *Trifolium* derive from agricultural AMF strains appears to attenuate over time (Lim & Cole 1984). Lastly, members of the genus are already known to adapt rapidly in response to changes in community biota and environmental conditions (Cocks & Phillips 1979; Hofmann *et al.* 2003; Olsen *et al.* 2008; Gilbert & Parker 2010). Most importantly, these species have a variety of geographic distributions in the non-native range

(that are not correlated with naturalisation date), allowing us to investigate whether post-naturalisation differences are correlated with species success.

Specifically, we make the following predictions:

1) Loss of soil mutualists will result in plants from non-native provenances having lower colonisation by rhizosphere mutualists (AMF and rhizobia) compared with plants from native provenances. We expect the magnitude of this difference to vary with soil origin:

    a) In native-range soils, where compatible mutualists are abundant but non-native plants have lost the ability to form associations, non-native plants will be poorly colonised whereas native plants will be well colonised.

    b) In non-native-range soils, where compatible mutualists may be scarce, plants from both native and non-native provenances will be poorly colonised.

2) AMF and rhizobia colonisation will be correlated, with low levels of rhizobia corresponding to low levels of AMF (evidence of "tripartite" benefit).

3) Plants from non-native provenances will have lower shoot-root ratios compared to native conspecifics, a trait that can compensate for reduced mutualist association.

4) The divergence in mutualist association between plants from native and non-native provenances will be positively correlated with species' geographic extents in New Zealand.

2.3 Methods

To test whether native and non-native populations differ in performance and mutualist association, we performed two glasshouse experiments using rhizosphere soil inoculants cultivated *in situ* by conspecifics in both the native range (Spain and the UK) and the non-native range (New Zealand). Glasshouse experiments were conducted in both the native and non-native ranges. Experiments with native-range soils were conducted at the Netherlands Institute of Ecology in Wageningen, The Netherlands, in Northern Hemisphere summer 2012. Experiments with non-native-range soil were carried out at Lincoln University in Canterbury, New Zealand, in Southern Hemisphere summer 2013.

2.3.1 Study species

We selected seven non-agricultural *Trifolium* species that were unintentionally introduced to New Zealand (Gravuer 2004): *T. arvense, T. campestre, T. glomeratum, T. micranthum, T. ornithopodioides, T. striatum* and *T. tomentosum* (Table 1.1). These annuals have each been naturalised in New Zealand for 84-160 years, suggesting sufficient time to adapt to the novel

rhizosphere communities of the non-native range (Atwood & Meyerson 2011). We specifically chose species with a range of regional (Figure 1.6) and countrywide (Figure 1.5) geographic distributions in New Zealand to test whether the magnitude of trait divergence could be correlated with naturalisation success. Data from a 1983-1988 vegetation survey (Wiser, Bellingham & Burrows 2001) of Banks Peninsula (see Pouteau, Hulme, & Duncan, 2014 for details) was used to estimate regional abundance of each species in the sampling area, Banks Peninsula, eastern Canterbury. Data from a Lincoln University Master's thesis (Gravuer 2004) was used for estimates of species abundance in the native-range and for New Zealand distribution. In each inter-provenance comparison, species serves as the unit of replication.

2.3.2 Study locations

For seed and soil collection in New Zealand we chose Banks Peninsula, Canterbury because it is the only region in New Zealand where all seven species co-occur with sufficient population densities for efficient soil collection and sufficient replication. In addition, this region comprises a variety of habitats broadly representative of the naturalised range of *Trifolium* on the South Island (Boswell *et al.* 2003) and the only location where all seven species co-occur with sufficient abundance for sampling with sufficient statistical power. Distribution records from a floristic survey conducted in the 1980s (Wilson 1992) assisted in the location of naturalised populations of each *Trifolium* species. We then selected two regions in the native range: the south coasts of England and Wales in the United Kingdom (UK); and northern Spain, from the Basque Country to Catalonia. The UK was chosen because it is the most likely source of European soil biota introduced to New Zealand, given these countries have an extensive history of trade and exchange of biological materials (Gravuer 2004). Botanical records from the Botanical Society of Britain and Ireland were used to locate British populations. Northern Spain was chosen to encompass a wider variety of native-range soil biota and to better match the latitude and climate in the non-native range. Three botanical databases were used to locate Spanish populations: Aranzadi (aranzadi.eus/botanica/herbario); Anthos (anthos.es); and records and personally communication with staff at the Jaca Herbarium, Instituto Pirenaico de Ecologia, Jaca, Spain. Specific locations in each country were chosen to encompass a wide range of climates and soil communities (see Appendix A for all sampling locations).

Ideally, tests investigating post-introduction adaptation compare plants from the founding population in the non-native range with the source population in the native range (Gundale *et al.* 2014), however this is rarely possible as the origins of most founding populations are unknown. For these *Trifolium* species, their native ranges encompass much of Europe, however many of New Zealand's agricultural clovers were imported from the UK (Gravuer 2004) making the UK a likely source location and an appropriate native-range comparison. To incorporate seed and soil from a

second region that more closely matched the sampling latitude in the naturalised range (Colautti *et al.* 2009), we also collected seed and soil in northern Spain. At all sites in both ranges, the species co-occurred with congeners, particularly the agricultural species *T. repens.*

2.3.3 Rhizosphere soil collection

Soil collection and storage methods were designed to capture as much of the rhizosphere microbiota as possible in each range. In each location and for each species, we collected soil at five soil locations—at least 1 km apart—in order to encompass a wide variety of soil types, land-use areas, aspects, elevations, and therefore soil communities. We collected approximately 100 mL of rhizosphere soil from beneath 10 plants located at least 1 m apart and placed the soil in separate bags. We sterilised our digging equipment between sites to avoid cross-contamination of soil biota. Soil collected from each site was air-dried (Reinhart *et al.* 2003), bulked and sieved to 4 mm. We also removed all visible macrobiota and roots before storing the soils in sealed bags in cool storage rooms (16-22°C).

2.3.4 Seed treatment

Seed was hand-collected from a minimum of 12 plants at one site for each species in each provenance (NZ, Spain, UK). Seed was tested for viability prior to the experiments. Field plants of *T. arvense* in the UK were not setting seed at the time of collection so we sourced UK seed from germplasm centre in the UK (Herbiseed). *T. tomentosum* was only sampled in New Zealand and Spain as it is not native to the UK, and *T. micranthum* and *T. ornithopodioides* were only sampled in New Zealand and the UK because we could not locate sufficient populations in Spain. Seed from a single site is not expected to encompass all the variation of a species in a particular provenance (Leger & Rice 2003; Erfmeier & Bruelheide 2005; Buschmann, Edwards & Dietz 2005; Blumenthal & Hufbauer 2007), however in this study, we wished to replicate at the species level to form inferences at a coarser scale. Although the study lacks population-level resolution, our study design gives us more predictive value because post-naturalisation differences that are common among multiple species may be indicative among a functional plant type (i.e. nitrogen-fixing legumes). To minimize the possibility of local seed adaptations (i.e. maternal effects) to site conditions or biota (Moloney *et al.* 2009), including rhizobia and AMF (Sherwood & Masterson 1974; Mytton 1975; Lie et al. 1987; Chanway, Holl & Turkington 1989; Porter *et al.* 2011), wherever possible the hand-collected seed was not taken from soil-collection sites (Appendix A).

All seed was treated to remove existing microbiota from the seed coat by sterilizing in a 10% solution of household bleach for 2 min and rinsing thoroughly in de-ionised water. Seeds were then scarified

gently with a scalpel to perforate the testa and germinated on sterile glass beads under species-specific temperature and day-length requirements in a germination cabinet (Appendix B.1).

2.3.5 Glasshouse experiments

To inoculate glasshouse pots with rhizosphere microbiota cultured by conspecifics from each range, we added a 10% (v/v) inoculum of unsterilised soil from a single site to the sterilised background soil in each 1 L pot. Using a fraction instead of the whole soil serves to minimise differences in abiotic soil properties (pH, macro- and micronutrient content, etc.) and also standardise the effect of nutrient flushes that occur after soil sterilisation. We mixed an unsterilised soil inoculum from a single soil site into each pot containing background soil that was sterilised by either by successive autoclaving (two cycles of 20 min at 121 °C) in New Zealand or by gamma irradiation ($\geq$25 kGray) in The Netherlands. Autoclaving did not appear to induce chemical changes damaging to plant growth and although this method of sterilising soil can alter soil structure, I suspect these differences to be minimal because we used sandy soils with only about 2-3% organic matter. Further, the organic matter and total nitrogen content of background soils were comparable between the two glasshouses (see Appendix B2).. Seedlings were transplanted into the glasshouses after emergence of the first true leaf. Seedlings that died within the first week were replaced. Pots were assigned random locations in the glasshouses, rotated every two weeks and watered to species-standardised weights on a weekly or twice-weekly basis as needed. During the experiments, we responded to outbreaks of thrips by releasing biocontrol mites *Amblyseius cucumeris* (twice in New Zealand and once in the Netherlands glasshouse) and we applied a topical, non-systemic fungicide (Chlorotek, NuChem, New Zealand) to all *T. campestre* plants in New Zealand to combat a glasshouse mould. Plants of the same species were harvested on the same day after approximately three months when plants began forming flower buds, indicating an energetic switch from growth to reproduction. Roots were washed gently, scored for rhizobia colonisation (details below) and each plant was separated into roots and shoots before being oven-dried at 65° C. We used growth rate (dry biomass / number of glasshouse-grown days) to standardize comparisons (McKenney *et al.* 2007). Roots and shoots were weighed separately to provide a shoot-root ratio (S:R).

2.3.6 Quantifying colonization by mutualists

Colonization of *Rhizobium leguminosarum* bv. *trifolii* was scored during root washing. We followed a modified protocol from Corbin et al. (1977), using a 0 to 3 scale that takes into account the number, size, location and colour of root nodules (Appendix C.1). Nodules that are pink to dark purple indicate the presence of leghaemoglobin, an oxygen-binding protein synthesised when rhizobia are actively fixing nitrogen to maintain anoxic conditions in the nodule (Somasegaran & Hoben 1985; Melino *et al.* 2012). Dark-coloured nodules near the base of the plant, larger than 1 mm and/or abundant

throughout the root system indicate the presence of beneficial nitrogen-fixing strains (Greenwood & Pankhurst 1977), whereas white or green nodules indicate lack of functionality or evidence of parasitism—bacteria benefiting from photosynthate without fixing nitrogen (Abd-Alla *et al.* 2014). Each plant might host as many as 10 or more strains, each of differing productivity (Denison & Kiers 2004a), so it is not possible to quantify the efficacy of nodules visually, however, nodule scores provide a proxy for the degree of rhizobial association, and our scoring guide allowed us to analyse performance as a function of association.

Arbuscular mycorrhizal fungi colonisation was quantified after plants were dried using an ink-and-vinegar protocol adapted from Vierheilig et al. (1998). First, we harvested oven-dried roots from the distal 2 cm of each plant and placed them in Eppendorf tubes in 70% ethanol to rehydrate them. Next, roots from each plant were placed in separate histology cassettes and heated in a 90° C bath of 10% KOH for 11 min to clear the cytoplasm. After rinsing with DI water, cassettes were transferred to a 5% solution of black Schaeffer ink in white vinegar and stained for 7 min at 80° C. To de-stain, we rinsed the cassettes several times in tap water and bathed them for at least 1 h in a room-temperature water bath acidified with a few drops of vinegar. Roots were plated on microscope slides using lactic acid and glycerol and examined under a compound microscope at 100x magnification. By moving the microscope stage in a horizontal plane, we scanned each slide at 1 mm intervals. When the centre of the viewing area intersected with root material, the material was scored as an arbuscule, vesicle, internal hyphae, external hyphae or root (Appendix C.2). Passes were made until we had 100 observations from each plant.

2.3.7 Statistical analyses

AMF and rhizobia mutualisms. To test whether rhizobia association is lower among naturalised populations we first compared the nodulation scores of plants from each seed provenance using a separate generalised linear mixed-effects (GLME) model for each soil provenance (NZ, Spain and UK). Each analysis was run separately in each native range soil allowing us to test for inter-provenance differences independently in each country. Species was treated as a random effect and seed provenance was designated a fixed effect. We accounted for potential non-independence due to site-specific effects by including the site from which soil was collected as a random effect. In each soil, we ran the models with and without the factor “seed provenance” and then used ANOVA to calculate the significance of seed provenance origin on nodulation score. We also tested whether rhizobia confer reduced growth benefit to naturalised populations, so we ran GLME models of growth rate as a factor of nodulation score. The regression slopes and uncertainties in these models were extracted to provide an estimate of the incremental growth-rate benefit of increasing the

nodulation score by one for each provenance, which we ran separately for each soil type. For simplicity, we refer to this value as the Rhizobium Mutualism Benefit (R-MB).

We also ran linear mixed-effects models for AMF colonisation. Models were run separately in each soil type with species and site designated random effects. Because our measure of colonisation was a count value, and count data can result in non-normal distributions and over-dispersion, we logit-transformed the data so we could use standard regression models with normal (Gaussian) errors. In each soil, we ran the model with and without the factor "seed provenance" and then compared the paired models using ANOVA to extract a significance value for the effect of seed provenance on AMF colonisation. To investigate the AMF growth benefit, we modelled the regression of growth rate as a factor of percentage colonisation with AMF (as explained above for rhizobia) which we term the AMF Mutualism Benefit (AMF-MB).

We fitted three regression models to examine the relationship between growth rate and mutualist colonization (percentage root infection for AMF and nodulation score for rhizobia), one for each soil origin (NZ, Spain and the UK). In NZ soil, the regression model included data from 89 pots: 7 species × 3 seed provenances × 5 replicates (with three species lacking provenances in one region and one missing value). In Spain and UK soils, the regression models included data from 47 and 60 pots, respectively. We used these data to test for differences between provenances in the slope of the relationship between growth rate and nodulation score. This meant that for each soil type, each provenance (NZ, Spain or UK) had between 23–34 observations on which the regression was fitted.

Tripartite associations. To investigate whether naturalised provenances have a lower level of "tripartite" associations (positive correlation of AMF and rhizobia within a single plant) compared to native conspecifics, we ran separate ANOVAs in each soil type with species and site designated random effects, seed provenance as a fixed effect, and using the dependent variables: percentage AMF, rhizobia score, the AMF-rhizobia interaction term, and the AMF-rhizobia-seed term (the latter to test for a difference in the tripartite benefit by seed provenance).

S:R ratios. To test whether plants from non-native provenances compensate for reduced mutualist association by diverting more energy to accumulating belowground biomass, we calculated shoot-root (S:R) ratios. For this analysis, we again built separate linear mixed-effects models for each soil with seed provenance as a fixed effect and species and site as random effects.

We used Pearson's correlations to test whether geographic distribution was correlated with the magnitude of inter-provenance differences in (i) rhizobia nodulation, (ii) AMF colonisation, (iii) MB values, and (iv) S:R ratios. In all analyses, species was the focal level of replication and the results are aggregated accordingly. The experimental design and statistical analyses were set up to allow us to

identify and quantify differences between provenances in the factors of interest—including differences in the opposite direction predicted. All statistical analyses were performed in R ver. 3.0.2 (R Development Core Team 2013) and model codes are detailed in Appendix E.2. Linear mixed-effects models were fit using the lmer function, which uses restricted maximum likelihood, in the R package "arm" ver. 1.6.10 (Gelman *et al.* 2014).

2.4 Results

2.4.1 Rhizobia nodulation and benefit

Nodulation scores among plants from both non-native and native provenances differed by species (Appendix D.1) and seed provenance origin (Figure 2.1), but in general were higher overall in native range versus non-native range soils. The mean nodulation scores (on a 0-3 scale, Appendix C.1) were 1.36 (± 0.07, S.E.) in New Zealand soils, 1.70 (± 0.08) in Spanish soils and 2.0 (± 0.11) in the UK soils. In addition, in the non-native range soils, 20% of all plants did not form functional nodules at all, whereas in native-range soils all plants but one formed functional nodules (Appendix D.3). Also in the non-native soil treatment, several plants from each provenance of two species, *T. glomeratum* and *T. tomentosum,* had nodules characteristic of parasitism (non-functional nodules are white or pale coloured). The low levels of nodulation for all provenances in New Zealand soil support our prediction that these soils lack the beneficial rhizobia strains of the native range. However, because glasshouse effects can confound differences between native and non-native range soils, all statistical analyses are restricted to inter-provenance differences within each soil origin.

As predicted, plants from non-native provenances had lower levels of rhizobia colonisation compared to native conspecifics. When grown in soils from the UK, plants from non-native provenances had a mean nodulation score of 1.77 (± 0.15 S.E.), compared to plants from the UK with 2.17 (± 0.14) ($F_{1,60}$ = 6.00; P = 0.02). Similarly, when grown in soils from Spain, plants from non-native provenances had a mean nodulation score of 1.67 (± 0.11) while plants from Spanish provenances had 1.74 (± 0.13), but this difference was not significant ($F_{1,47}$ = 0.20; P = 0.64) (Figure 2.1). Although nodulation was generally low for all seed provenances in the New Zealand soil treatment, plants from non-native provenances still had significantly lower rhizobia nodulation scores in New Zealand soils compared to plants from native provenances. The mean nodulation score for non-native plants was 1.09 (± 0.11) compared to 1.53 for both Spanish (± 0.14) and UK (± 0.13) provenances ($F_{2,139}$ = 4.57; P = 0.03) (Figure 2.1).

In addition to having lower rhizobia colonisation, the performance benefit, defined as the growth-rate increase as a function of nodulation score (R-MB), was significantly lower among plants from non-native provenances compared to native conspecifics in all soils (Figure 2.2). The biggest inter-

provenance difference was observed in the Spanish soil comparison, where plants from the Spanish provenance had an R-MB that indicated 9 mg/d greater increase in growth per level of nodulation relative to non-native plants ($F_{1,47}$ = 10.39; P < 0.01) (Figure 2.2). R-MB values for each species are in Appendix D.5.

Not surprisingly, non-native plants also had significantly lower growth rates (Figure 2.3). The growth difference was most extreme in New Zealand soils where, averaging across all species, the mean growth rate of plants from non-native provenances was 37% lower than native conspecifics ($F_{1,139}$ = 15.13; P = 0.002). In native-range soils, plants from naturalised populations grew an average of 26% slower than plants from Spanish populations soil ($F_{1,47}$ = 18.49; P < 0.001) and 22% slower than plants from UK populations ($F_{1,60}$ = 10.16; P < 0.04).

2.4.2 AMF association and benefit

Colonisation by arbuscular mycorrhizal fungi (AMF) varied among species, with an average colonisation level of between 11% and 54% (Appendix D.1). Total colonisation levels were highly varied and only one inter-provenance difference was significant—UK plants had higher colonisation than New Zealand plants in New Zealand soil (Figure 2.4). However, in both native-range soil treatments plants from native provenances had significantly higher AMF-MB scores (Figure 2.5). As with the R-MB scores, the inter-provenance divergence in AMF-MB was highest in Spanish soil ($F_{1,47}$ = 9.33; P < 0.01). Species-level AMF-MB values are detailed in Appendix D.4.

2.4.3 Evidence for "tripartite" associations

As predicted, there was a significant positive relationship between rhizobia nodulation and colonisation by AMF in every soil type (Appendix E.3), The regressions showed that increasing nodulation values correlated strongly with increasing AMF percentages, evidence for the "tripartite" association among *Trifolium,* rhizobia and AMF. All three ANOVAs (run separately for each soil origin) had P values of <0.05 for the amf-nods interaction variable (Appendix E.2). In addition, in the Spanish soil treatment, there was also a significant AMF-rhizobia-seed interaction, suggesting that in Spanish soil, the Spanish and New Zealand seed provenances differed in their tripartite association (Appendix E.2B); with New Zealand provenances having the expected positive association and Spanish provenances having a slight negative correlation (at higher nodulation scores, Spanish plants had lower AMF colonisation).

2.4.4 S:R ratios

Shoot-root ratios (S:R) showed greater allocation to above-ground biomass among all plants, and S:R values were not significantly different between native and non-native provenances within a soil

origin, except for in UK soil, where plants from non-native provenances had an average S:R of 2.8 (± 0.21 S.E.) and native UK provenances S:R was 2.3 (± 0.15) ($F_{1,60}$ = 6.01; P < 0.02) (Figure 2.6), which was the opposite of what is predicted if plants from non-native provenances compensate more for lost root mutualists with greater allocation to root growth.

2.4.5 Geographic extent

Overall, there were no trends between the magnitude of the inter-provenance differences in mutualism association and species' regional and countrywide geographic distributions in New Zealand. Only two of these correlations were statistically significant and they were in opposite directions—there was a negative correlation between the inter-provenance difference in rhizobia nodulation and countrywide geographic distribution in Spanish soil (Cor = -0.92, P = 0.03) and there was a positive correlation between the inter-provenance difference in rhizobia nodulation and NZ geographic distribution in UK soil (Cor – 0.81, P = 0.05). Because plants from New Zealand provenances had significantly lower growth rates, we also looked for a correlation between growth rate differences and species geographic distributions; but none of these correlations was significant.

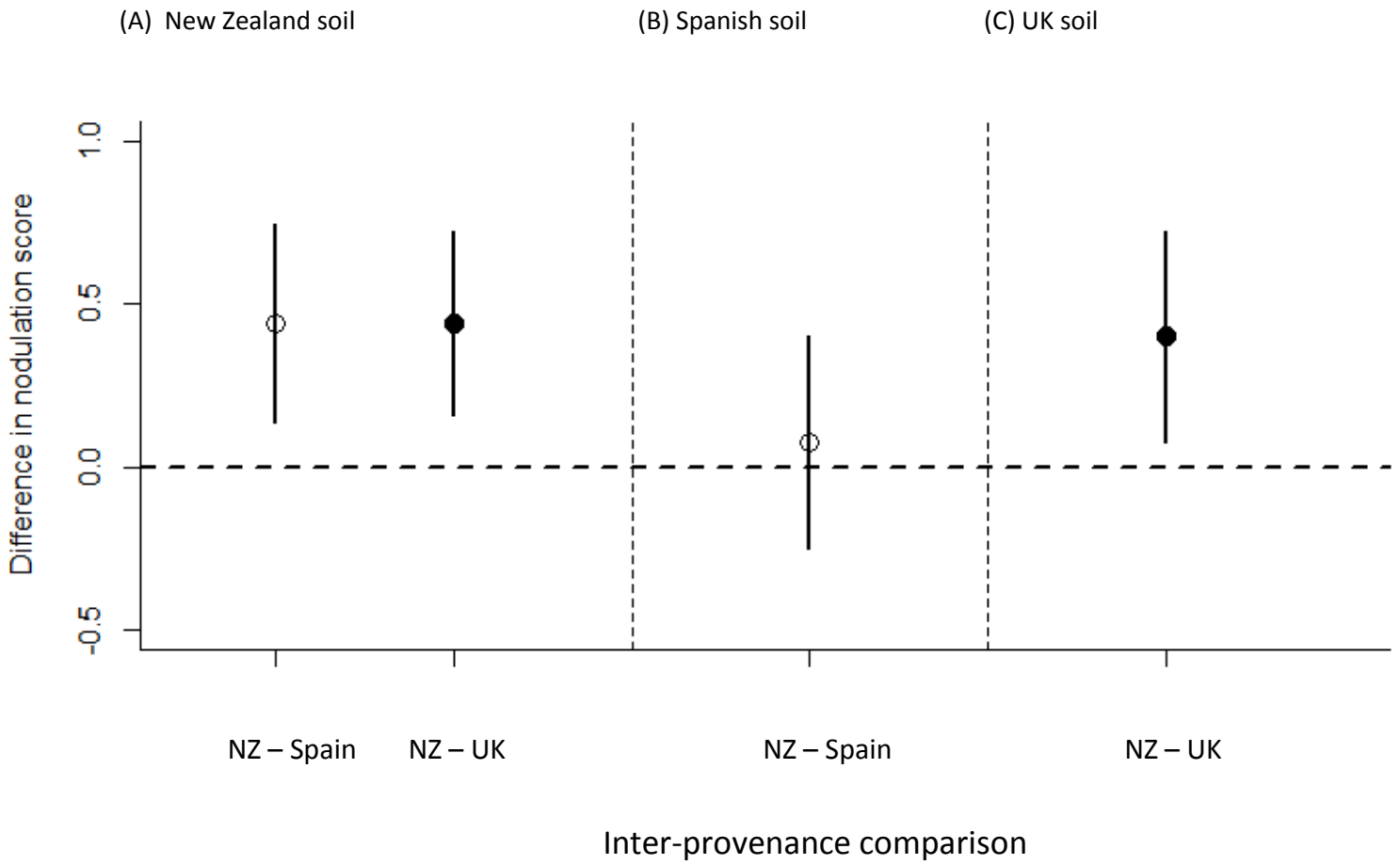


Figure 2.1. Mean difference in nodulation score between non-native (New Zealand) and native (Spain = ○ and UK = ●) provenances of seven species of *Trifolium* grown in unsterilised rhizosphere soils cultured by conspecifics in the non-native range (NZ) and native range (Spain and the UK). Points are a reference class in which plants from native provenances are compared to plants from non-native provenances. Thus, zero would represent no difference between provenances and points above the line signify that nodulation was higher among plants from the native provenance. Nodulation was measured via a 0-3 nodulation score (Appendix C.1). Bars are 95% confidence intervals extracted from the model coefficient tables. Linear mixed-effects models were run separately by soil. Full model details and ANOVA output are in Appendix E.

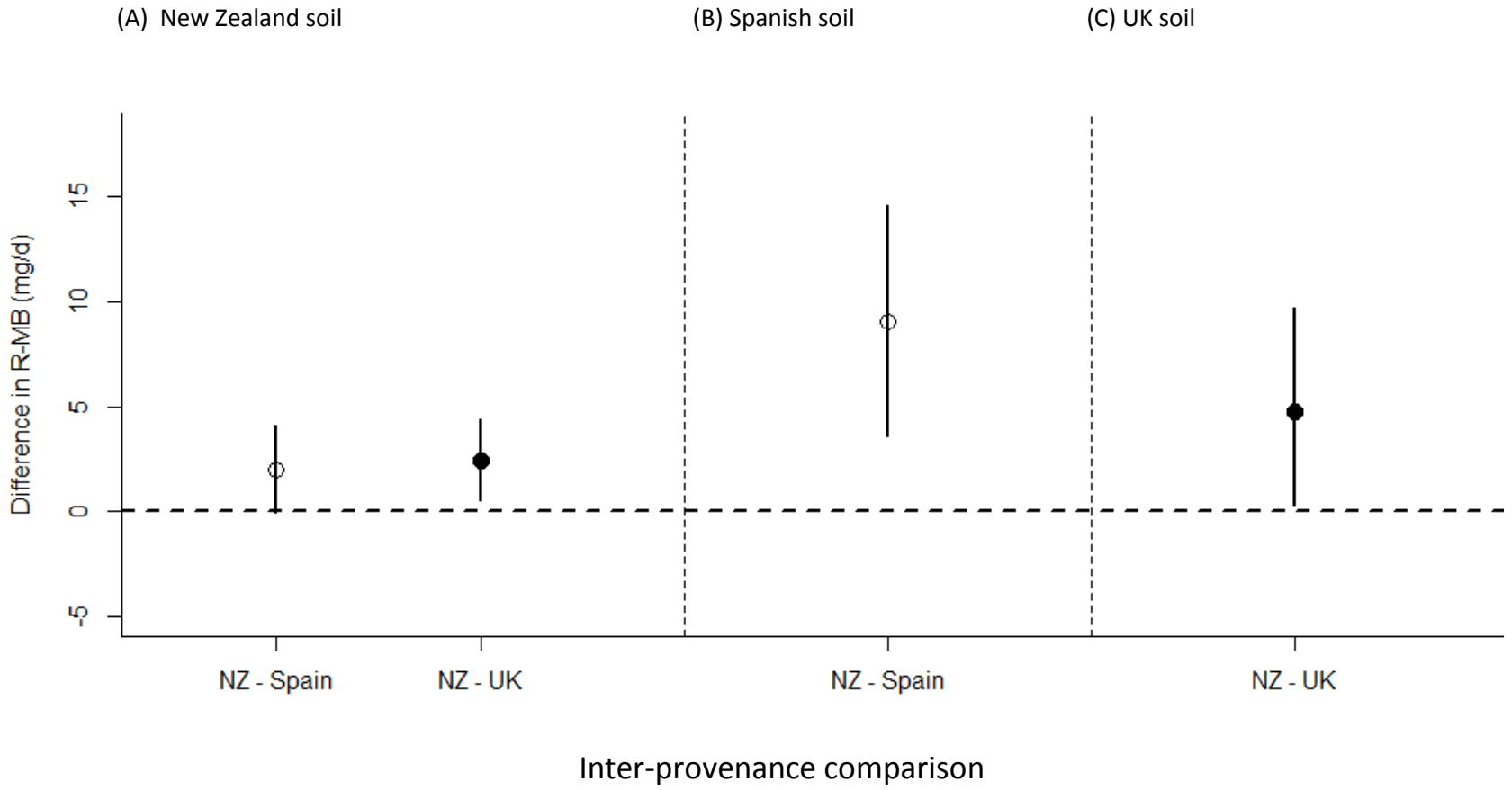


Figure 2.2. Mean difference in rhizobia mutualist benefit (R-MB) in mg dry-weight growth per glasshouse day between non-native (New Zealand) and native (Spain = ○ and UK = ●) provenances of seven species of *Trifolium* grown in unsterilised rhizosphere soils cultured by conspecifics in the non-native range (NZ) and native range (Spain and the UK). Points are a reference class in which plants from native provenances are compared to plants from non-native provenances. Thus, zero would represent no difference between provenances and points above the line signify that nodulation was higher among plants from the native provenance. Nodulation was measured via a 0-3 nodulation score (Appendix C.1). Bars are 95% confidence intervals extracted from the model coefficient tables. Linear mixed-effects models were run separately by soil. Full model details are in Appendix E.

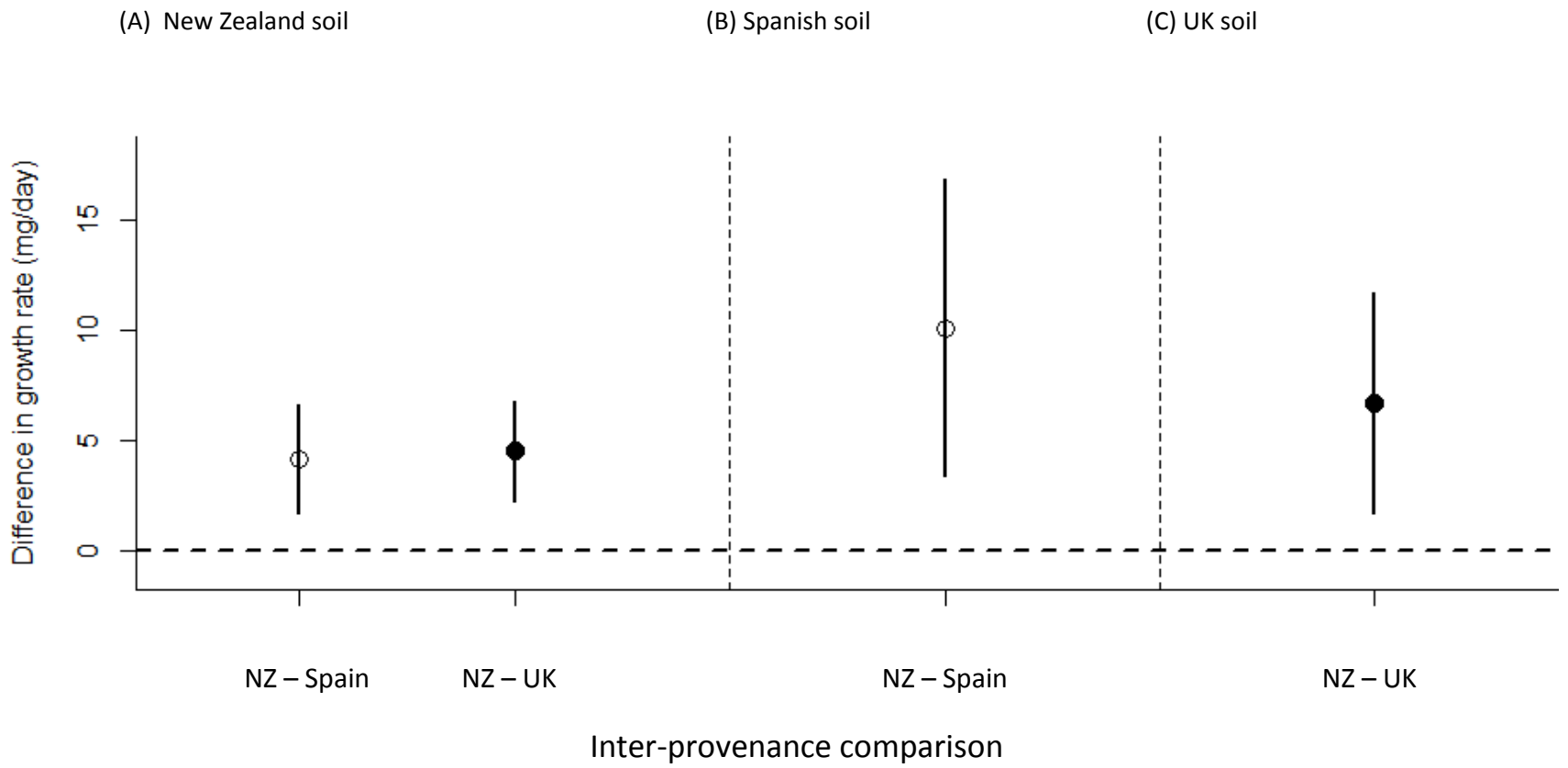


Figure 2.3. Differences in growth rates between non-native (New Zealand) and native (Spain = ○ and UK = ●) provenances of seven species of *Trifolium* grown in unsterilised rhizosphere soils cultured by conspecifics in the non-native range (NZ) and native range (Spain and the UK). Points are a reference class in which plants from native provenances are compared to plants from non-native provenances. Thus, zero would represent no difference between provenances and points above the line signify that growth was higher among plants from the native provenance. Bars are 95% confidence intervals extracted from the model coefficient tables. Linear mixed-effects models were run separately for each soil type. Full model details and ANOVA output are in Appendix E.

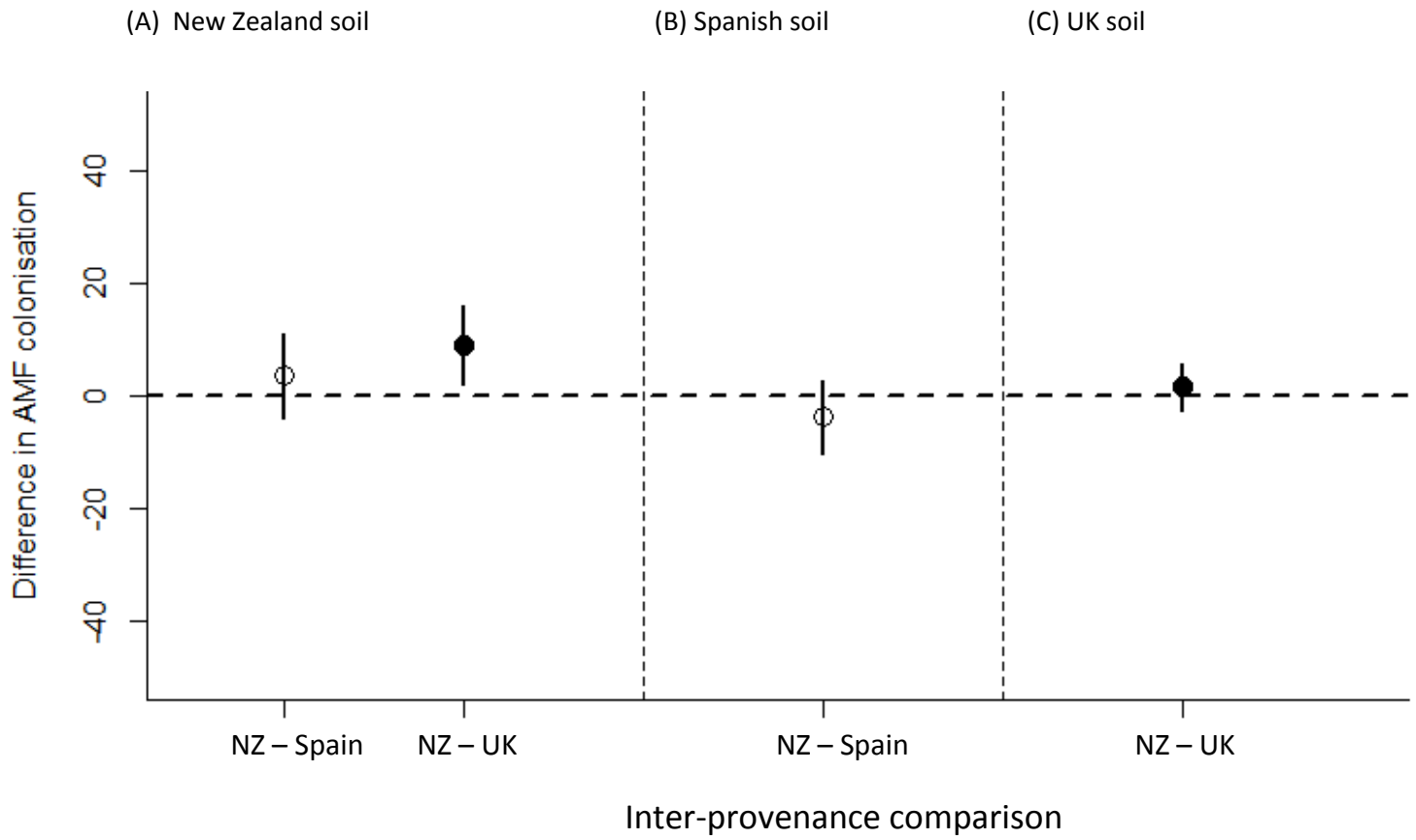


Figure 2.4. Differences in colonisation by arbuscular mycorrhizal fungi between non-native (New Zealand) and native (Spain = ○ and UK = ●) provenances of seven species of *Trifolium* grown in unsterilised rhizosphere soils cultured by conspecifics in the non-native range (NZ) and native range (Spain and the UK). Points are a reference class in which plants from native provenances are compared to plants from non-native provenances. Thus, zero would represent no difference between provenances and points above the line signify that colonisation was higher among plants from the native provenance. . Colonisation was measured as a percentage (Appendix C.2). Bars are 95% confidence intervals extracted from the model coefficient tables. Linear mixed-effects models were run separately for each soil type. Full model details and ANOVA output are in Appendix E.

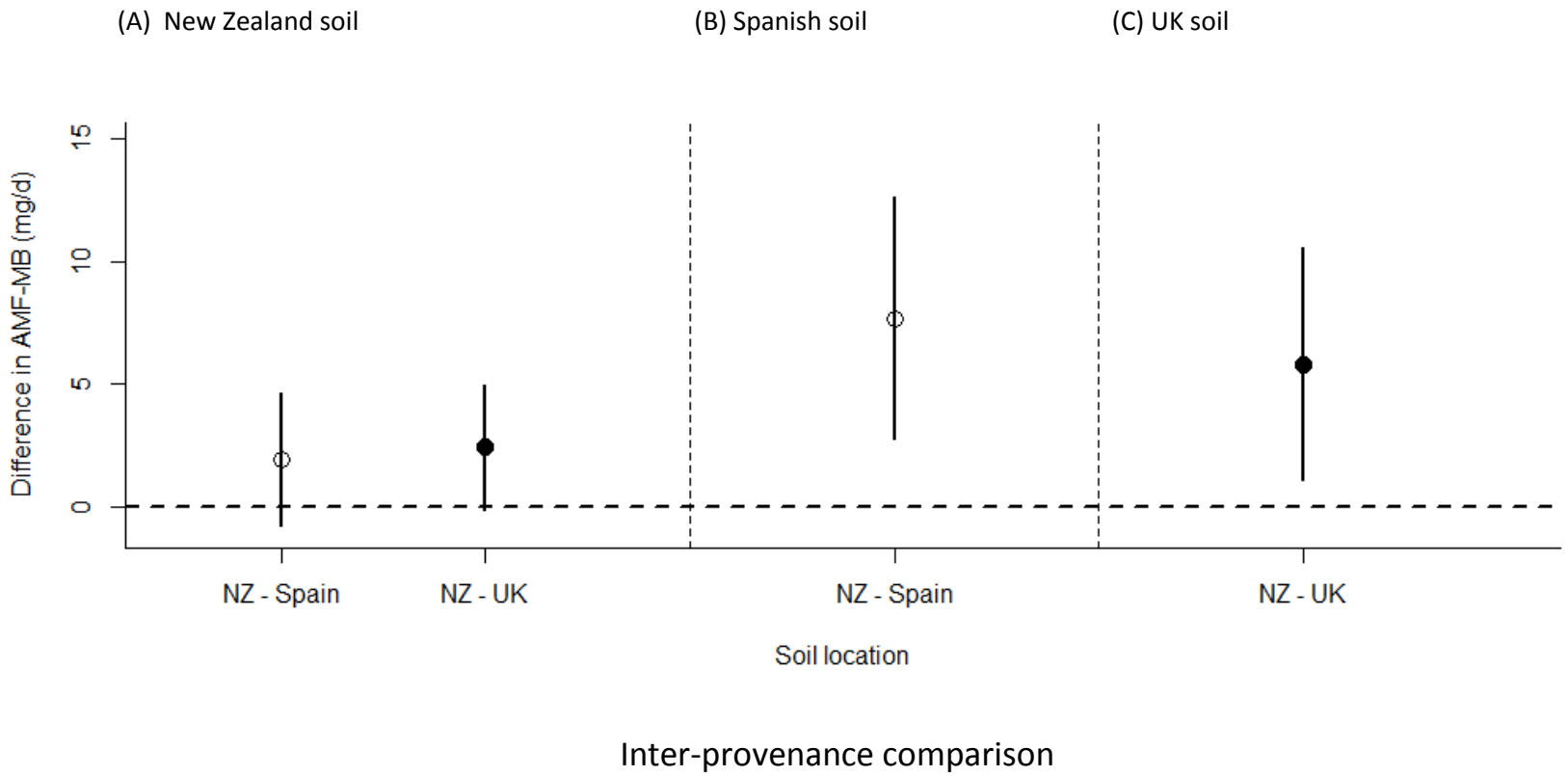


Figure 2.5. Mean difference in AMF mutualist benefit (AMF-MB) in mg dry-weight growth per glasshouse day between non-native (New Zealand) and native (Spain = ○ and UK = ●) provenances of seven species of *Trifolium* grown in unsterilised rhizosphere soils cultured by conspecifics in the non-native range (NZ) and native range (Spain and the UK). Points are a reference class in which plants from native provenances are compared to plants from non-native provenances. Thus, zero would represent no difference between provenances and points above the line signify that the growth benefit derived from AMF was higher among plants from the native provenance. Colonisation was measured as a percentage (Appendix C). Bars are 95% confidence intervals extracted from the model coefficient tables. Linear mixed-effects models were run separately by soil. Full model details are in Appendix E.

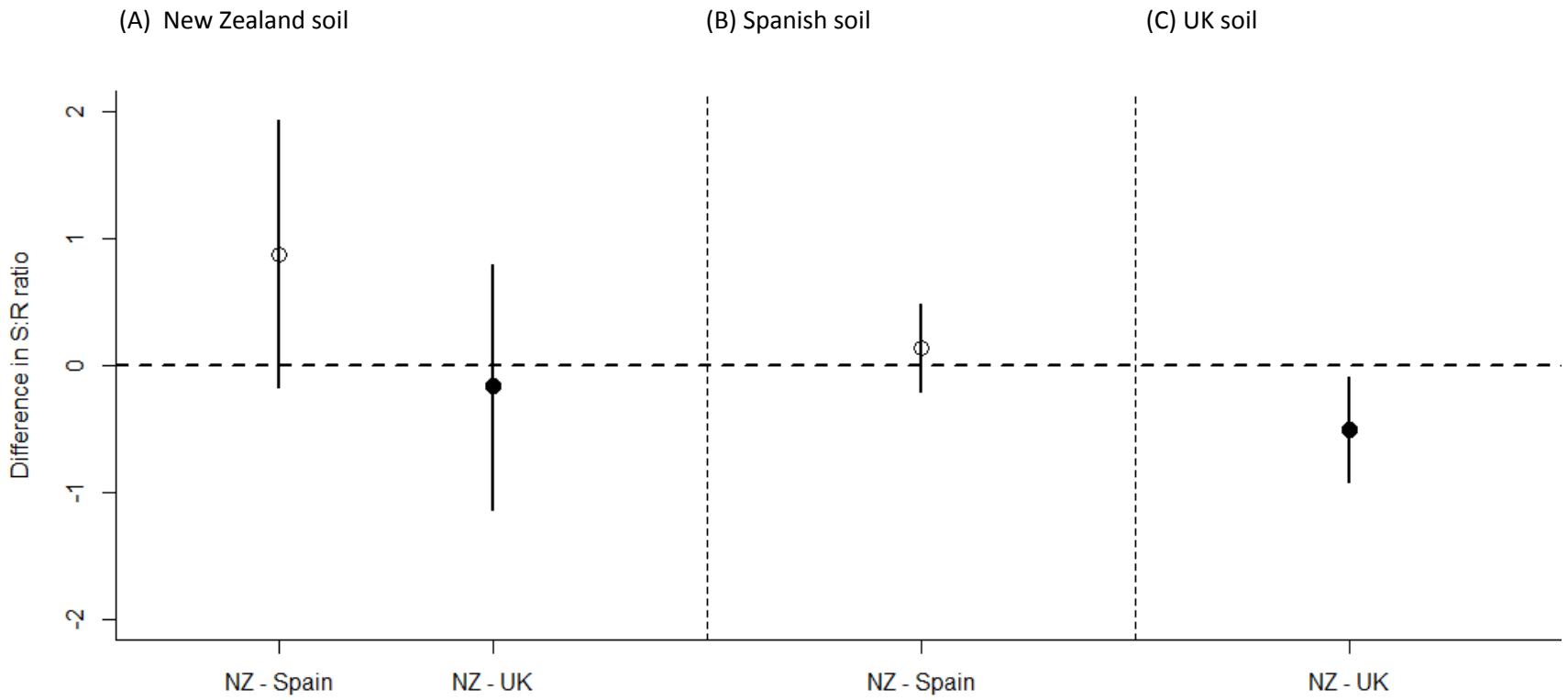


Figure 2.6. Differences in shoot:root ratios (S:R) between non-native (New Zealand) and native (Spain = ○ and UK = ●) provenances of seven species of *Trifolium* grown in unsterilised rhizosphere soils cultured by conspecifics in the non-native range (NZ) and native range (Spain and the UK). Points are a reference class in which plants from native provenances are compared to plants from non-native provenances. Thus, zero would represent no difference between provenances and points above the line signify that shoot:root ratios were higher among plants from the native provenance. Lower S:R ratios are common among non-mycorrhizal plants. Bars are 95% confidence intervals extracted from the model coefficient tables. Linear mixed-effects models were run separately for each soil type. Full model details and ANOVA output are in Appendix E.

2.5 Discussion

In this study, *Trifolium* from non-native provenances had lower rhizobia colonisation than native conspecifics—even when exposed to native-range rhizobia and when grown in rhizosphere soils that enabled native conspecifics to form abundant functional nodules. Association with arbuscular mycorrhizal fungi was varied, but also significantly lower among non-natives in the Spanish soil treatment. In addition, the growth benefit of associating with both mutualists was lower among plants from non-native provenances. However, the inter-provenance differences in mutualist association were not correlated with species geographic distributions. Moreover, *T. glomeratum* plants from New Zealand were the only plants completely lacking nodulation in New Zealand soils, yet this species is among the most widespread in the country (Figure 1.5) and the most common of the seven species in the New Zealand study area (Figure 1.6). Thus for non-native *Trifolium* in New Zealand, rhizosphere mutualist associations appear to be reduced, but this does not appear to be a factor in naturalisation success.

2.5.1 Reduced mutualist association

The low levels of rhizobia nodulation in New Zealand soils among plants from all provenances further supports surveys that show that annual *Trifolium* from Europe do not benefit greatly from the naturalised agricultural strains of rhizobia in New Zealand (Howieson *et al.* 2005). The lower nodulation by non-native plants in all soils may be evidence for adaptation to reduced mutualists in the non-native range. Potential explanations that may explain the reduced mutualist association include: (i) the rhizobia available to annual *Trifolium* in New Zealand are not as beneficial as the rhizobia they co-evolved with in the native range; (ii) nitrogen is not a limiting nutrient in the environment these plants colonise; and (iii) the protective mechanism incurred by rhizobia colonisation is not important in the non-native range.

The first explanation (reduced benefit) is supported in this study by the lower overall nodulation in New Zealand soils and by our observation of parasitic nodules only in the New Zealand soils. In New Zealand soils, some plants from all three provenances of two species, *T. glomeratum* and *T. tomentosum*, had nodules that were pale or white, characteristics associated with rhizobia that are taking photosynthate from plant vascular tissue, but not fixing nitrogen (Appendix C.1). Each nodule on a plant's root system is colonised independently and multiple rhizobia strains frequently colonise a single nodule (Denison 2000), thus, parasitic strains can co-occur with beneficial strains on a single plant or within a single nodule (Long 1989). While completely non-functional or parasitic nodules can be identified by eye, the presence of ineffective strains in heterogeneous nodules is much more difficult to assess, even with genetic analyses (Yates *et al.* 2005). Parasitism would certainly form a strong selective pressure against the rhizobia mutualism in the non-native range and could also explain why many annual *Trifolium* have failed to

naturalise in New Zealand (Gravuer 2004). The mutualism-parasitism continuum is an aspect of invasion research that deserves a closer look, as parasitic relationships are common when resources are rich (Karst *et al.* 2008). It has been suggested that plant invaders face a higher incidence of parasitism in their non-native range (Thrall *et al.* 2007a) but that this phenomenon goes unnoticed simply because it is masked by other benefits (e.g. concurrently higher resource levels, enemy release or reduced competition) in the non-native range (Lockwood *et al.* 2007; van der Putten *et al.* 2007).

Evidence for the second explanation (nitrogen is not a limiting nutrient), may be supported by the observation that in all of our sampling locations in the non-native range, species of the target *Trifolium* species co-occurred with at least one congener, usually *T. repens, T. dubium* or *T. subterraneum.* Rhizobia-hosting legumes exude excess nitrogen into the soil, providing this limiting nutrient to plants nearby—the basis of their benefit to pastoral agriculture. The annual clovers used in this study were never found in the absence of agricultural congeners and this, along with their reduced rhizobia association, suggests that these species may be relying on proximity to agricultural species in the absence of their own highly compatible rhizobia strains in the non-native range. This explanation is further supported by the apparent lack of physiological compensation among non-native plants. Although plants from non-native provenances were significantly smaller, their shoot-root ratios were similar compared to native conspecifics, whereas shoot-root ratios that are smaller indicate energetic reallocations to roots in order to acquire more nutrients or water.

The third potential explanation (reduced need for protection) is informed by the results of a parallel study (McGinn 2015) that used plant-soil feedback experiments to show that several of these annual *Trifolium* species are benefitting from enemy release from negative rhizosphere biota in New Zealand. Although AMF and rhizobia each provide limiting nutrients, their colonisation can also be a protective barrier against belowground pests and pathogens, including nematodes (Philippot *et al.* 2013). If *Trifolium* in New Zealand are enjoying release from enemies, the protective aspects of the mutualism may not be needed and this may also contribute to reduced mutualist dependency without negatively affecting naturalisation.

2.5.2 Reduced growth among non-native plants

Significantly lower growth rates were exhibited by non-native *Trifolium* compared to native conspecifics in all soil types (Figure 2.3), and this difference may be at least partly attributable to the lower association with AMF and rhizobia, and the lower incremental growth benefits of these associations. Other studies document smaller *Trifolium* in their non-native ranges (Boswell *et al.* 2003), and in some cases this is believed to be a physiological response to drought or UV stress (Hofmann *et al.* 2003). A comparison of plant sizes from published floras suggests that at least leaflet lengths are smaller among non-native

Trifolium in both New Zealand and California, compared to the UK and Spain (Appendix D.2) (Webb, Sykes & Garnock-Jones 1988; CSIC 2015). We reject the idea that observed differences are plastic because this study only compared plants from native provenances to plants from non-native provenances in the same soil treatment, and models were run separately for each soil treatment. Smaller size among non-native *Trifolium* could be a founder effect, however size was significantly smaller across seven species, which suggests that the smaller sizes are a post-naturalisation response to environmental or biotic conditions in the non-native range. These may include the effects of soil nutrients, UV levels, water availability, competition with heterospecific plants, herbivory, or any number of soil pests and pathogens that compose the rhizosphere biota.

Although smaller size and slower growth rates would seem to contradict the performance characteristics commonly associated with invaders (Lockwood, Hoopes & Marchetti 2007), a study of multiple invasive plant families in New Zealand showed that seedling relative growth rate alone was not correlative of invasive ability (Bellingham et al. 2004) and it has been suggested that the reason size has been historically misconstrued as an inherent characteristic of invaders is because the field data on invader size has consisted of non-random sampling (i.e. larger, problematic invaders are more likely to be studied) (Bossdorf *et al.* 2005). In addition, reduced size and slower growth rates can be a protective mechanism against drought, temperature stress and low nutrient availability (Buswell *et al.* 2011). Of these factors, low nutrient availability may be the most relevant in the *Trifolium* system in New Zealand, as soils are generally phosphorus and nitrogen poor (Crush 1982; Maxwell 2013) and decreased access to nutrients could be compounded by the unavailability of ideal rhizosphere endophytes, which would normally assist in the uptake of nutrients from the soil. Although in this study we controlled for the effect of nutrient differences among soil-sampling locations by using only 10% (v/v) whole-soil inocula in the glasshouse pots, nutrient differences between the native and non-native ranges could be driving differences in size (Zhang & Jiang 2006).

2.5.3 Conclusion

This work provides multi-species evidence of reduced rhizosphere mutualist association and benefit among seven non-native species of a globally successful genus. Parasitism by available rhizobia in the non-native range may be a contributing factor to decreased association and benefit. The lack of evidence for physiological compensation and the lack of correlation between the divergence in mutualist association/benefit and geographic distribution suggest that mutualist availability is not a factor in the naturalisation success of these seven *Trifolium* species.

Chapter 3

Root flavonoid production reduced among non-native *Trifolium* in native-range soils

3.1 Abstract

Plant-microbe interactions in the rhizosphere are mediated by exchanges of complex secondary metabolites—including flavonoids, which act as hormones to enhance root colonisation by beneficial endophytes or as defences to repel antagonistic biota. When plants colonise outside their native range, they inevitably encounter new rhizosphere communities and this may result in changes to the bio-active compounds they produce. The evolution of increased competitive ability (EICA) hypothesis posits that non-native plants displaced from co-evolved antagonists down-regulate production of defences, enabling them to reallocate energy toward becoming more competitive—one explanation for why some plants become invasive. Although EICA has found some support aboveground when plants escape native-range herbivores and down-regulate foliar defences, it has not been tested in the context of rhizosphere microbial communities, which contain both antagonists and mutualists. To investigate the role of rhizosphere microbiota and plant origin on root flavonoid production, we performed glasshouse experiments using seed from native (Spain and the UK) and non-native (New Zealand) provenances of five non-agricultural *Trifolium* species. Pots were inoculated with a 10% fraction of either unsterilised (i.e. composed of rhizosphere microbial communities) or sterilised rhizosphere soil cultivated *in situ* by conspecifics in each range. After three months, root flavonoid content was analysed by HPLC. Flavonoid richness and concentration were lower among non-native plants in native-range soil, as predicted by the EICA hypothesis. Specifically, richness and concentration of daidzein—an isoflavonoid relevant to both the mutualism with nitrogen-fixing rhizobia and defence from soil antagonists—was generally lower in native-range soils. Differences in flavonoid production between plants from native and non-native provenances were most pronounced in the sterilised native-range soils, where non-native plants produced about two-thirds the number of flavonoids of native conspecifics. Decreased flavonoid production in the presence of native-range biota may be evidence for loss of rhizosphere mutualists and/or enemies, however we found no evidence for performance trade-offs and thus EICA is only partially supported. This work contributes to a growing number of studies that find evidence for shifting biochemical profiles among invaders in the non-native range; however the lack of correlation with geographic spread suggests these differences are not a factor in the naturalisation success of these species.

3.2 Introduction

The rhizosphere—the soil region immediately surrounding plant roots—is densely colonised by microbiota that affect plant performance and competitive ability. Plants interact with these microbes via complex, often taxon-specific exchanges of secondary metabolites (Weston & Mathesius 2013). Rhizosphere microbial communities differ among plant communities (Coats & Rumpho 2014) and geographic locations (Pringle *et al.* 2009; Andonian *et al.* 2012; Nuñez & Dickie 2013; Tedersoo *et al.* 2014), thus, when a plant establishes outside its native range it will inevitably encounter novel rhizosphere microbial communities, which may lack co-evolved antagonists or mutualists. Previous studies have shown that soil-borne microbes in the native range are generally negative to plant growth (Willis *et al.* 2000; Mitchell & Power 2003; Torchin *et al.* 2003; Gundale *et al.* 2014) whereas those in the non-native range tend to have neutral or even growth-promoting effects (Inderjit & van der Putten 2010), suggesting plants benefit from escaping co-evolved antagonistic biota. The evolution of increased competitive ability (EICA) hypothesis predicts that in response to enemy release, plants will down-regulate production of defence compounds, and funnel energy toward other fitness traits that enable them to be more competitive and possibly even more successful post-naturalisation (Blossey & Nötzold 1995).

Most studies of EICA have focussed on release from aboveground herbivores and the down-regulation of foliar defences, such as alkaloids, tannins and terpenes (Maron *et al.* 2004a; Hull-Sanders *et al.* 2007; Ridenour *et al.* 2008; Franks, Wheeler & Goodnight 2011). In the rhizosphere, one group of compounds likely to be under selective pressure are flavonoids (Bais *et al.* 2004; Cooper 2007; Bhattacharya, Sood & Citovsky 2010), which function as both as toxic defence compounds against soil-borne antagonists (Sabudak & Guler 2009) and as hormone-like signals with soil-borne mutualists (Dakora & Phillips 1996; Andersen & Markham 2006). Some flavonoids are constitutive (i.e. produced independent of stimuli) and some are synthesised in response to specific stimuli (Tahara 2007; Sisa *et al.* 2010; Hassan & Mathesius 2012), such as the presence of a microbial chemical signature, triggering production of specific combinations and concentrations of flavonoids that inhibit or enhance interactions with that microbe (Walker *et al.* 2003; Bais *et al.* 2003; Popovici *et al.* 2011). A subset of root flavonoids are responsible for inducing, maintaining or rejecting colonisation by root mutualists, such as arbuscular mycorrhizal fungi (AMF) (Nair *et al.* 1991; Wright *et al.* 1998a; Osmond 1999; Ene & Alexandru 2008) and nitrogen-fixing rhizobia (Chatel & Greenwood 1973; Miller *et al.* 2007; Yates *et al.* 2008).

In this study, we test whether the production of flavonoids differs among non-native plants in the introduced range compared to conspecifics from the native range, using *Trifolium* (Fabaceae), a genus that is globally widespread. We predicted that plants from non-native provenances would have constitutively lower root flavonoid richness (i.e. lower production even in the absence of biotic stimuli) compared to

native conspecifics as a result of naturalising in a region lacking many, if not most, of the co-evolved rhizosphere antagonists and mutualists of the native range. We test this prediction by using sterilised soil treatments. Further, we expected to see lower flavonoid richness in plants from non-native seed provenances (compared to native seed provenances) when grown in unsterilised rhizosphere soil cultivated *in situ* by conspecific plants in the native-range. Because the non-native range is likely to lack these species' co-evolved rhizosphere microbiota, we predicted that root flavonoid production would be low and similar for all plants (native and non-native) when grown in New Zealand soils cultivated *in situ* by conspecifics. We also assessed the production of a specific isoflavonoid associated with belowground defence and mutualisms in many Fabaceae species. Daidzein (4',7-dihydroxyisoflavone) is a precursor to coumestrol and both of these isoflavonoids function as phytoalexins—biological toxins produced in high concentration to inhibit microbes, specifically fungal pathogens (Zilliken *et al.* 1984). Daidzein is also a phytoanticipin—stored in cells in anticipation of attack (Dakora & Phillips 1996)—and thus informs on constitutive root flavonoid production. Importantly, daidzein is considered the key flavonoid in nodulation initiation in the legume-rhizobia mutualism (Stafford 1997; Cooper 2007). In a parallel study (Chapter 2), we found rhizobia nodulation was significantly lower among New Zealand-naturalised plants, so we also wished to test if a positive correlation exists between rhizobia nodulation and flavonoid production. We tested the following specific hypotheses:

1. As a result of reduced contact with co-evolved antagonistic biota and highly compatible mutualist strains, plants from non-native provenances produce fewer flavonoids compared with native provenances both in sterilised soil (independent of biotic stimuli) and when grown in rhizosphere soil containing microbes cultivated *in situ* by conspecifics in the native range.

2. As an integral signal in the rhizobia mutualism, richness and concentration of daidzein flavonoids is positively correlated with rhizobia colonisation.

3. Plants from non-native provenances, which have reduced association with mutualistic rhizobia compared to native provenances (Chapter 2) and may be experiencing release from rhizosphere antagonists (McGinn 2015), produce fewer daidzein-like compounds and have lower daidzein concentrations.

3.3 Methods

3.3.1 Study species

This study used five non-agricultural *Trifolium* species *: T. arvense, T. glomeratum, T. ornithopodioides, T. striatum* and *T. tomentosum* (Appendix F.4). Species are all annuals and have been naturalised in New

Zealand for between 84-145 years (Appendix F.4), suggesting sufficient time for selection to occur in response to a novel environment (Atwood & Meyerson 2011). Like many Fabaceae, *Trifolium* produce numerous flavonoids (Kazakov, Litvinenko & Ammosov 1973; Polasek, Queiroz & Hostettmann 2007) and the common agricultural species, *T. repens,* down-regulates flavonoid production in favour of increased growth in some environments (Hofmann & Jahufer 2011). We chose non-agricultural species because we hypothesized that the production of flavonoids related to the mutualism with its nitrogen-fixing rhizobia, *Rhizobium leguminosarum* bv. *trifolii*, may be under strong selection. Naturalised rhizobia optimal for non-agricultural *Trifolium* are believed to be scarce in New Zealand soils (Hastings *et al.* 1966) because soils are dominated by strains for agricultural clovers (Nangul *et al.* 2013) even far from where they are seeded (Denton *et al.* 2003). In addition, in a parallel study found that association with and benefit from rhizobia is reduced among these species (Chapter 2). In this study, species is the intended level of replication, as each *Trifolium* species has its own suite of optimal rhizobia mutualists and rhizosphere antagonists, and thus each species forms an independent comparison between the performance of plants from the native and non-native provenances.

3.3.2 Seed and soil collection and glasshouse experiments

For seed and soil collection in the non-native range, we selected Banks Peninsula, Canterbury, a region encompassing a variety of habitats broadly representative of the naturalised range of *Trifolium* on the South Island of New Zealand (Boswell *et al.* 2003)—and the only location where all five species co-occur with sufficient abundance for sampling with sufficient statistical power. We then selected two regions in the native range (northern Spain to broadly match the latitude in the naturalised range and the southern United Kingdom because this is the source country for ~85% of the agricultural *Trifolium* species in New Zealand (Gravuer 2004) (Appendix A.1). Soil collection and storage methods were designed to capture and maintain the viability of as much of the rhizosphere microbiota as possible. At each of five sites in each region, we collected approximately 100 mL of rhizosphere soil from beneath 10 plants of each species. Sites were at least 1 km apart to encompass a variety of soil types, land use areas, elevations, aspects and therefore soil communities. Rhizosphere soil samples were located at least 1 m apart and placed in separate bags. Digging equipment was sterilised between sites with bleach or Dettol to avoid cross-contamination of soil biota. An equal fraction of each of the 10 soil samples collected from each site was air-dried (Reinhart *et al.* 2003), the samples were bulked, thoroughly mixed and sieved to 4 mm. We also removed all visible macrobiota and roots before storing the soils from each site in separate, sealed bags in cool storage rooms (16–22 °C). Half of the bulked soil from each site was sterilised by successive autoclaving (two cycles of 20 min held at 121 °C) in New Zealand and by gamma irradiation (>25 kGray) in the Netherlands. The sterilised soil treatments were intended as a control to separate flavonoid production

in response to rhizosphere microbiota (unsterilised treatments) and in the absence of rhizosphere microbial communities from each range (sterilised treatments). Autoclaving did not appear to induce chemical changes damaging to plant growth and although this method of sterilising soil can alter soil structure, I suspect these differences to be minimal because we used sandy soils with only about 2-3% organic matter. Further, the organic matter and total nitrogen content of background soils were comparable between the two glasshouses (see Appendix B2).

Seed was hand-collected from plants at one site in each region (NZ, Spain, the UK) from a minimum of 12 plants, pooled, cleaned and tested for viability prior to the experiments. Field plants of *T. arvense* in the UK were not setting seed at the time of collection so we sourced seed for the native provenance of this species from a germplasm centre in the UK (Herbiseed). *T. tomentosum* was only sampled in New Zealand and Spain as it is not native to the UK, and *T. ornithopodioides* was only sampled in New Zealand and the UK. To avoid maternal effects (Moloney *et al.* 2009) (i.e. that seed provenances were locally adapted to soil microbiota (Sherwood & Masterson 1974; Mytton 1975; Lie et al. 1987; Chanway, Holl & Turkington 1989), wherever possible the hand-collected seed was not taken from soil-collection sites (Appendix A.1-A.2). Seeds were treated to remove microbiota from the seed coat by sterilizing in a 10% solution of household bleach for 2 min and rinsing thoroughly in de-ionised water. Seeds were then scarified gently with a scalpel to perforate the testa. Seeds were germinated on sterile glass beads under species-specific temperature and day-length requirements in a germination cabinet (Appendix B.1).

To inoculate glasshouse pots with microbiota cultured by conspecifics from each range, we added a 10% (v/v) inoculum of rhizosphere soil (either sterilised or unsterilised) from a single site to a sterilised background soil in each 1 L pot. Using a fraction instead of the whole soil serves to standardise the effect of nutrient flushes from the sterilised inocula and to minimise differences in abiotic soil properties (pH, macro- and micronutrient content, etc.). No fertilizers or soil amendments were used (Appendix B.2). There were 5 replicate pots in each of two treatments (sterilised and unsterilised inocula for each of the 5 soil sites for each species in each country). With 5 species, 3 seed provenances, and 3 soil origins, this gave a total of 300 pots. (N.B. The design is not fully factorial because UK seed was only grown in UK and NZ soils and Spanish seed was only grown in Spanish and NZ soils.). Seedlings were transplanted into the glasshouses after emergence of the first true leaf. After transplant, the exposed soil of each pot was covered by aluminium foil to minimise contamination by microbiota among the pots. Pots were blocked by treatment (sterilised versus unsterilised), assigned a random location in the glasshouses and rotated between blocks and positions within each block every two weeks. All plants were watered from below (to avoid contamination from splashing) to a species-standardised weight on a weekly or twice-weekly basis as needed. Plants of the same species were harvested on the same day after three months when plants began

forming flower buds, indicating an energetic switch from growth to reproduction. Plant roots were gently washed and scored for rhizobia nodulation (Appendix C.1) before sections were harvested for flavonoid analysis.

3.3.3 HPLC root sample preparation and flavonoid isolation

Root pieces were selected from the distal ~5 cm of each plant's root mass. Rhizobia nodules were excluded from cuttings as we wished to analyse only the plant-produced flavonoid production. Root material was removed with sharp scissors and immediately placed into labelled vials and stored at -80° C until they were freeze-dried under pressure for 36–48 h. Freeze-drying is considered the least destructive method for flavonoid extraction because plants are subjected to desiccation stress for less time than in a drying oven and there is less exposure to flavonoid-degrading UV light (Zainol 2009). Only plants with sufficient root material (dry weight > 10 mg) were used for HPLC flavonoid analysis, and thus the total number of replicates was 221 (with no discernible bias by seed provenance origin or treatment)—108 in the sterilised treatment and 113 in the unsterilised treatment.

Freeze-dried roots from each plant were individually weighed, placed in micro-centrifuge tubes, pulverised to a fine powder and their cell contents extracted in methanol (1 mg powder to 20 mL of 70% methanol) at room temperature for 2 h using a high-speed oscillator followed by vortex. The material was then centrifuged at 1500 rpm, at 4 °C for 20 min. A 50 µL aliquot of supernatant was removed from each tube and diluted with 450 µL of 100% methanol. Aliquots were transferred to amber-tinted vials to reduce flavonoid degradation during processing. The extraction protocol was based on previous work (Ingham & John L 1978; Polasek *et al.* 2007; Prati *et al.* 2007) and optimized to provide ideal expression and isolation of flavonoid peaks. The high-performance liquid chromatography (HPLC) methodology was developed and validated for each experimental run according to ICH requirements for specificity, linearity, accuracy, precision (repeatability and intermediate precision) as outlined in Shabir (2004).

3.3.4 HPLC materials and equipment

All standard stock solutions were of > 95% purity and sourced from Fisher Scientific (NZ) or Sigma Aldrich (NZ). The acetonitrile and methanol were HPLC grade and the acetic acid was analytical grade. Water was purified on a Milli-Q system (Sartorius Stedim Biotech, NZ). The analyses were performed on an Agilent 1100 series HPLC machine using a C18 4.6 x 150 mm Kinetex column (Agilent, Santa Clara, CA, USA). Each experimental run consisted of a single species of *Trifolium* with seed provenance fully randomised. Each run was preceded by two "blanks" composed of pure methanol and each sample was preceded by a needle wash to prevent contamination among samples. Each sample injection was composed of a 10 µl aliquot. Flow rate was 0.5 mL/min, column temperature was set to 40 °C and we recorded absorbance at 210 nm,

260 nm and 320 nm. Samples were run using five gradient time steps of two solvents over a total run of 30 min. Solvent A was 0.01% acetic acid in Milli-Q water and Solvent B was 0.01% acetic acid in acetonitrile (full details in Appendix G). Purchased standards of six flavonoids common to *Trifolium* were run in concentrations of 5, 10, 20 and 50 ppm for each experiment to create calibration curves. All curves had R-squared values of > 0.95. The 20 ppm standard stock mix was run at the beginning and end of each experimental run as a control. In runs involving more than 25 samples, this 20 ppm mix was also quantified in the middle of the run. In addition, one randomly selected root sample was run in triplicate during each run to confirm repeatability.

3.3.5 Flavonoid analyses

Flavonoid peak identities were validated by both retention time (within 0.1 min of the purchased chemical standards) and each flavonoid's characteristic UV spectral pattern at 260 nm using Agilent ChemStation chromatography software (1100 series). This software displays the spectrum of each flavonoid and also calculates a numerical value to characterise the degree of similarity between the spectrum of a particular peak and the reference spectrum in the spectral library. In our analysis, to be considered a match for a particular standard, we required that the UV pattern of a peak match that of the chemical reference standard with greater than 85% similarity. This method allows for the identification of flavonoid peaks for quantitative analysis in the absence of a mass detector, but does not allow identification of spectra for which there is no reference standard. Quantification (total flavonoid concentration in parts per million, ppm) was calculated on the basis of a standard curve of daidzein; daidzein was chosen for the validation process because it was the most abundant flavonoid identified in all five species of *Trifolium*.

3.3.6 Study assumptions

The exact mechanisms of root-microbe flavonoid exchanges has been difficult to elucidate as a result of (i) variability in inhibition/stimulation thresholds by compound, concentration, timing and taxa (Dakora & Phillips 1996), (ii) difficulties detecting flavonoids, which can function at nanomolar concentrations (Weston & Mathesius 2013), and (iii) challenges sampling *in situ,* as exudates transform and are quickly adsorbed by soil (Hassan & Mathesius 2012). In this study, we only analysed flavonoids located in the distal portions of roots, where flavonoid production is believed to be most active in response to rhizosphere microbiota (Weston & Mathesius 2013). We also made two assumptions regarding the structure of the rhizosphere communities in the glasshouse. First, although we did not analyse the microbial diversity in the glasshouse pots, we assume that the unsterilised soil treatments harbour microbiota representative of each range. This assumption is supported by previous analyses (Chapter 2) showing that plants from native-range provenances are colonised by both arbuscular mycorrhizal fungi and nitrogen-fixing rhizobia in both native

and non-native unsterilised soil treatments. Second, we assume that although sterilised soil does not remain completely biota free in any glasshouse (Reinhart & Callaway 2006), that these treatments remained relatively biota-free and that the opportunistic microbes that did colonise the sterilised pots represent only a fraction of the community found in the unsterilised inocula (W. van der Putten, per. comm.); thus the sterilised treatment allows us to estimate constitutive metabolite production—i.e. production of flavonoids in the absence of at least many of the specific signals produced by rhizosphere microbial communities.

3.3.7 Statistical analyses

Total flavonoid concentrations, flavonoid richness, concentrations of the isoflavonoid daidzein and rhizobia colonisation of native and alien plants were analysed using linear mixed effect models (Appendix F.1). Each analysis was run separately in each native range soil allowing us to test for inter-provenance differences independently in each country. For analyses of flavonoid concentration, concentration (ppm) data were log-transformed for normality. Species and site were designated random effects; seed provenance was a fixed effect. Models were run separately for each soil type. To control for over-dispersion in the flavonoid richness data (a count value that represents the number of flavonoid peaks in each plant sample), we created an additional random-effect variable that took a random value for each of the observations in the dataset and then fit each model with a Poisson distribution. To test for a significant difference between seed provenances in each analysis, models were run with and without the fixed factor "seed provenance" and the results were compared by ANOVA. Pearson's product moment correlations were used to test for a significant correlation between level of rhizobia colonisation and the concentration and richness of the isoflavonoid daidzein. Pearson's correlations were also used to test for a significant correlation between the difference in native plant versus non-native plant production of flavonoids in each species and the extent of that species' regional and countrywide geographic distributions (Appendix F.4). The experimental design and statistical analyses were set up to allow us to identify and quantify differences between provenances in the factors of interest—including differences in the opposite direction predicted. All statistical analyses were performed in R ver. 3.0.2 (R Development Core Team 2013). Linear mixed-effects models were fit using the lmer function, which uses restricted maximum likelihood, in the R package "arm" ver. 1.6.10 (Gelman *et al.* 2014).

3.4 Results

3.4.1 Flavonoid richness

Flavonoid richness was lower among plants from non-native provenances compared to native conspecifics in native-range soils and this difference was significant in both native-range sterilised soils (Spain: $F_{1,27}$ =

10.17, P = 0.003; UK: $F_{1,25}$ = 14.88, P < 0.001) (Figure 3.1A) and in the UK unsterilised soil ($F_{1,24}$ = 10.99, P = 0.007) (Figure 3.1B). In both the New Zealand soil treatments (sterilised and unsterilised), flavonoid richness was similar among all three provenances and not statistically different (sterilised: $F_{1,56}$ = 0.52, P = 0.34; unsterilised: $F_{1,63}$ = 0.02, P = 0.76). Cumulatively, in the sterilised treatments, the mean number of unique flavonoids produced by non-native plants in native-range soil was about 2/3 the number produced by native conspecifics (Appendix F.5). However, there were no significant correlations between the magnitude of difference in flavonoid richness between provenances and species geographic distribution on a regional or countrywide scale.

3.4.2 Flavonoid concentration

In both the native-range soil treatments (and in both sterilised and unsterilised), total flavonoid concentrations (in parts per million, ppm) tended to be lower among plants from non-native provenances compared to either of the conspecifics from Spain or the UK (Figure 3.2), although in sterilised NZ soil, UK plants had a lower concentration than either Spanish or NZ plants. Across all soil origins and treatments, New Zealand root flavonoid concentrations were about 4/5 that of native conspecifics, however the results of the mixed-model analysis showed that the only soil treatment in which NZ plants had significantly lower flavonoid concentration compared to native conspecifics was in the UK sterilised soil treatment ($F_{1,25}$ = 4.79, P = 0.03) (Figure 3.2). Again, differences between provenances were not correlated with species' regional or countrywide geographic distributions.

3.4.3 Daidzein and rhizobia nodulation

In unsterilised soil from both native-range provenances and in unsterilised soil from the non-native-range, root daidzein richness was significantly, positively correlated with rhizobia nodulation (Appendix F.6). All Pearson's correlation coefficients were greater than 0.45 and all P values were less than 0.01. Daidzein concentration, however, was only positively correlated with rhizobia nodulation in the unsterilised New Zealand soil treatment; in the two native-range soils there was a minor and non-significant negative correlation (Appendix F.6).

3.4.4 Daidzein richness and concentration

In soils from the native range, daidzein concentration and richness were generally lower among plants from New Zealand (Figure 3.3, Figure 3.4; Appendix F.5), however the difference was only significant in one comparison—concentration of daidzein in the UK sterilised soil treatment (P = 0.03) (Figure 3.4). This was in contrast to expectations because daidzein and rhizobia were strongly positively correlated (Appendix F.6) and an analysis of rhizobia nodulation in the unsterilised treatment (Appendix F.3) showed that nodulation

was generally lower among plants from New Zealand, and significantly lower when New Zealand plants were compared to UK provenances, for these five species. In soils from the non-native range, daidzein concentration and richness were similar and not significantly different (Figure 3.3, Figure 3.4).

3.4.5 Flavonoid-biomass trade-offs

We found no evidence for the predicted negative correlation between root biomass and flavonoid concentration (R^2 = 0.019). Species, our unit of replication, differed substantially in these regressions—in three species root biomass and flavonoid concentration were positively correlated, while the other two species had a negative correlation (Figure 3.5A). In addition, seed provenances differed significantly in the root biomass-flavonoid regressions, with plants from New Zealand and Spanish provenances having a slight negative association between flavonoid production and root biomass, as would be expected, but UK provenances having a slight positive association (Figure 3.5B).

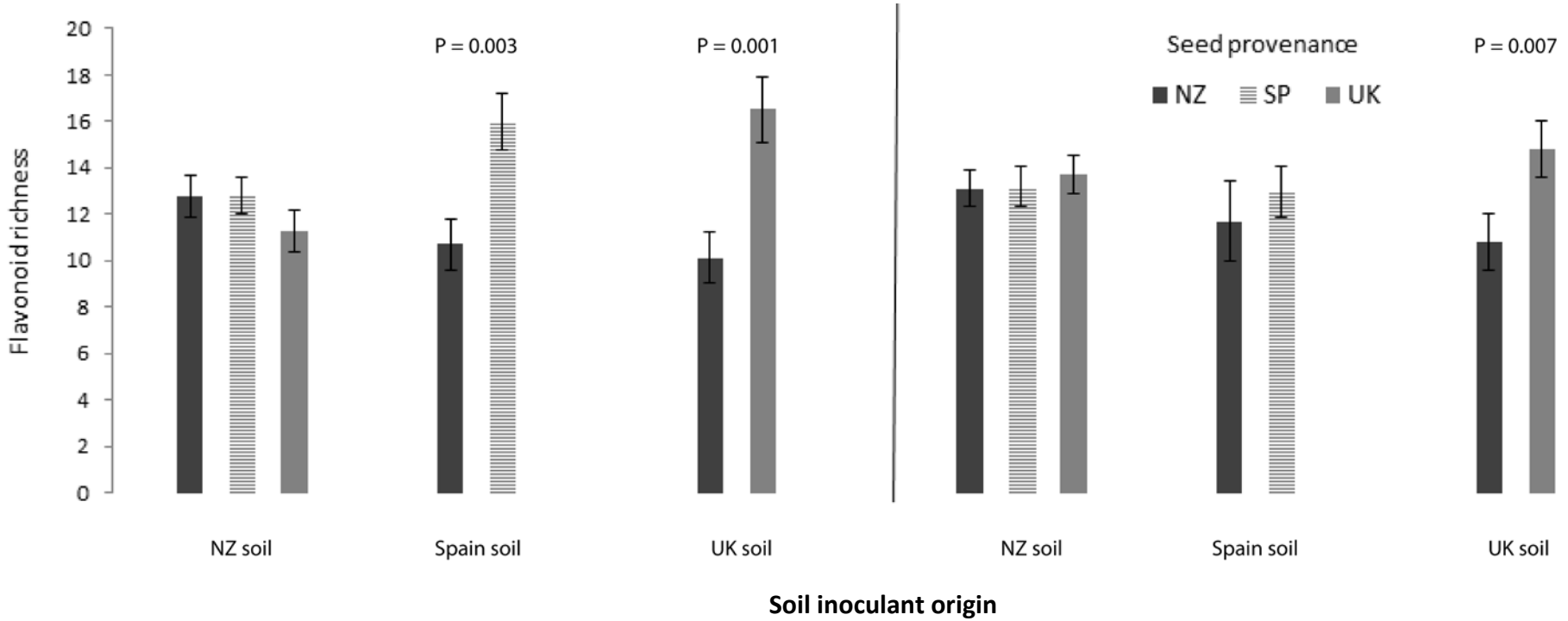


Figure 3.1. Root flavonoid richness (total number of flavonoids) of plants from each of the three seed provenances (New Zealand, NZ; Spain, SP; and the UK) for five species of *Trifolium* grown in (A) sterilised and (B) unsterilised soils from the non-native (New Zealand) and native (Spain and the UK) ranges. Bars are S.E.M. across all species. Significance values were extracted from the linear mixed-effects models.

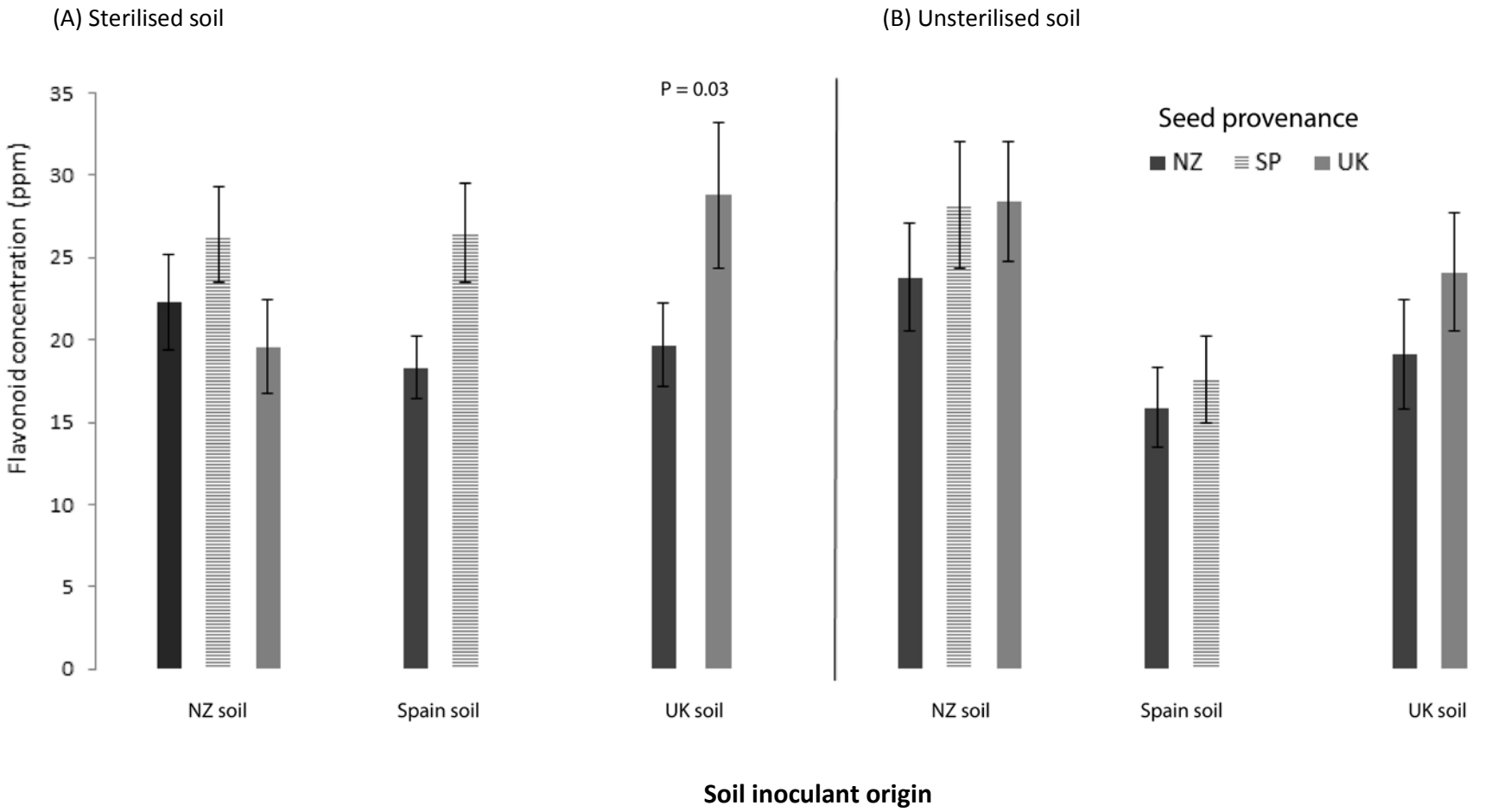


Figure 3.2. Root flavonoid concentration (ppm) of plants from each of the three seed provenances (New Zealand, NZ; Spain, SP; and the UK) for five species of *Trifolium* grown in (A) sterilised and (B) unsterilised soils from the non-native (New Zealand) and native (Spain and the UK) ranges. Bars are S.E.M. across all species. Significance values were extracted from the linear mixed-effects models.

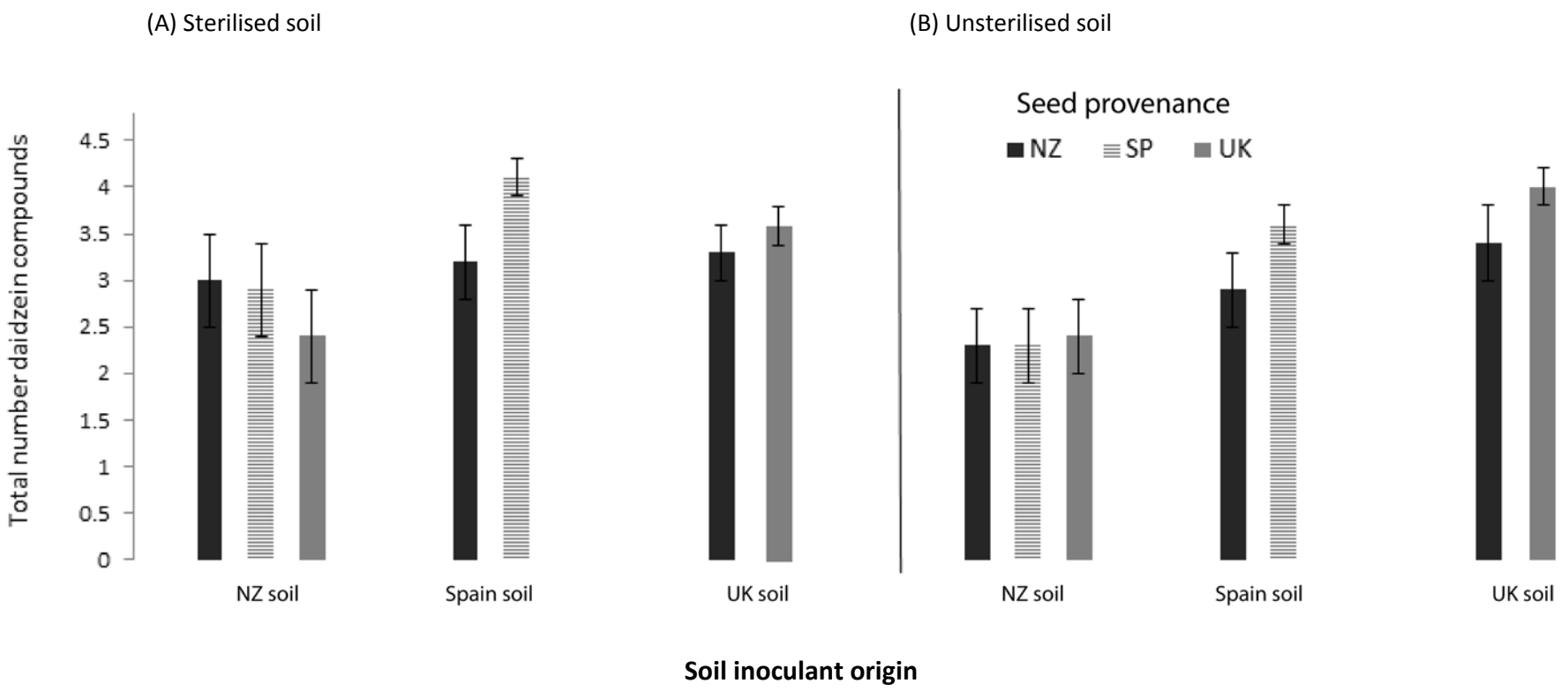


Figure 3.3. Root daidzein richness (number of daidzein compounds) of plants from each of the three seed provenances (New Zealand, NZ; Spain, SP; and the UK) for five species of *Trifolium* grown in (A) sterilised and (B) unsterilised soils from the non-native (New Zealand) and native (Spain and the UK) ranges. Bars are S.E.M. across all species. Significance values were extracted from the linear mixed-effects models.

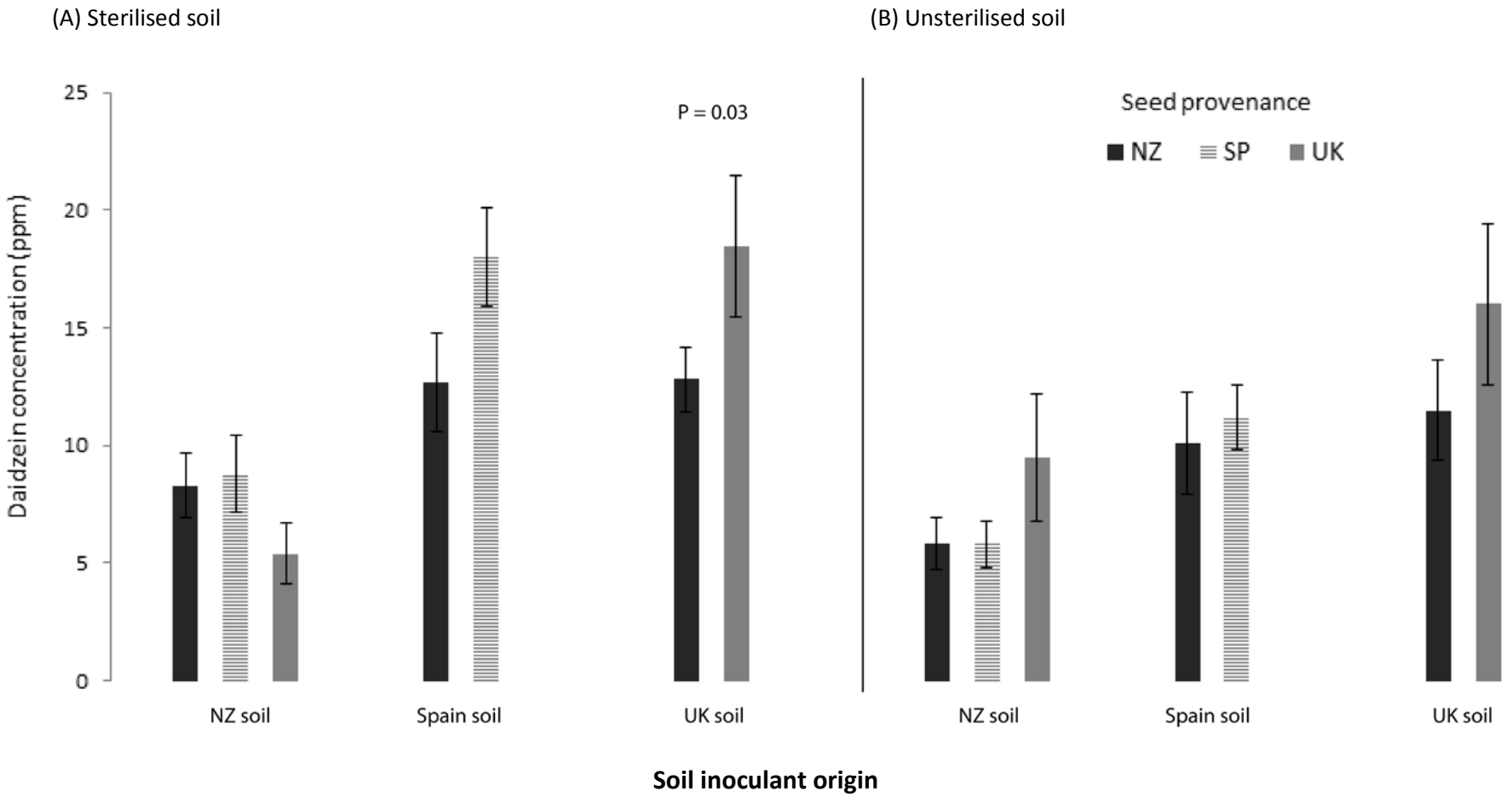
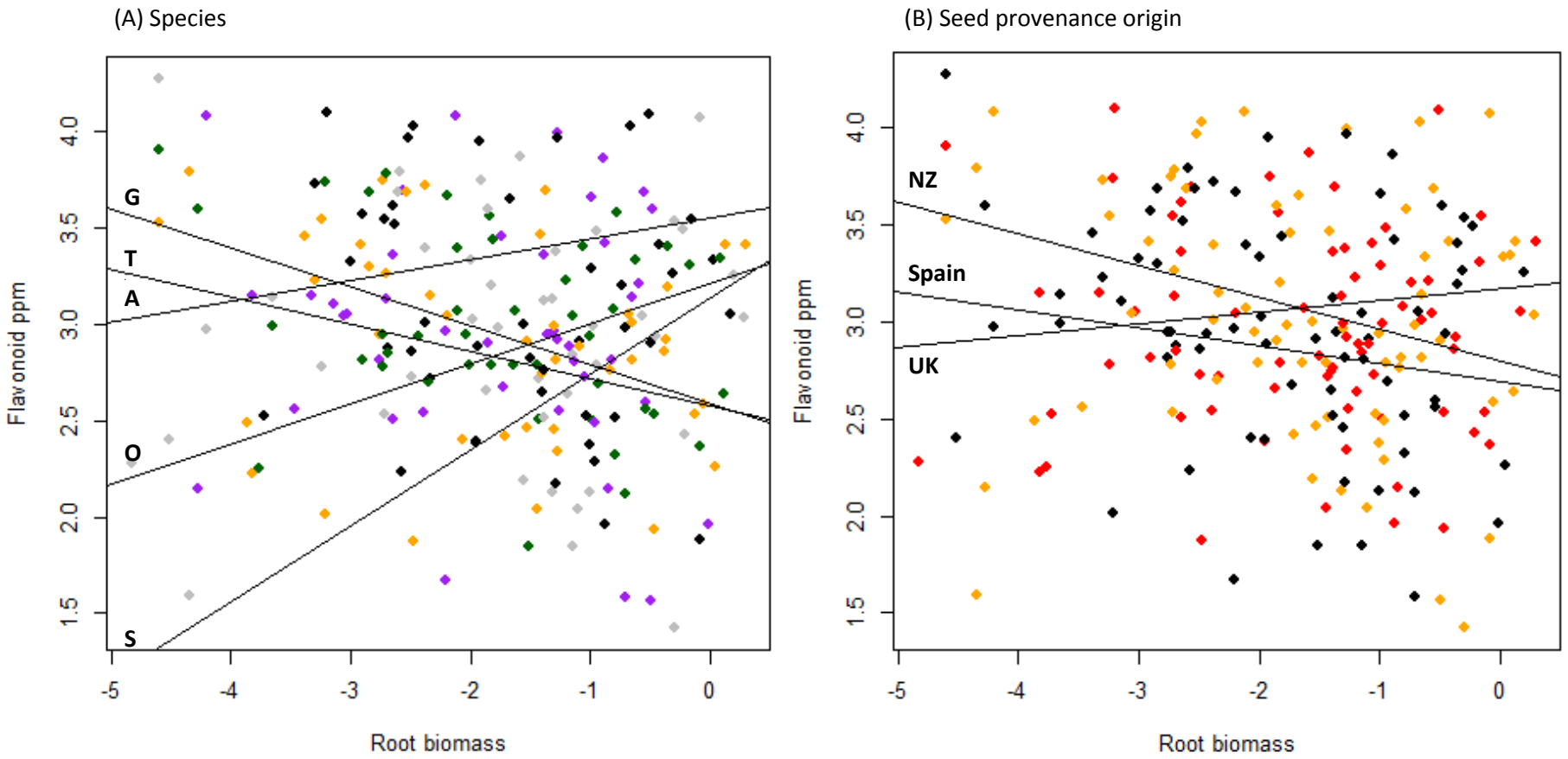


Figure 3.4. Root daidzein concentration (ppm) of plants from each of the three seed provenances (New Zealand, NZ; Spain, SP; and the UK) for five species of *Trifolium* grown in (A) sterilised and (B) unsterilised soils from the non-native (New Zealand) and native (Spain and the UK) ranges. Bars are S.E.M. across all species. Significance values were extracted from the linear mixed-effects models.



(A) Species = *T. arvense* (A, green), *T. glomeratum* (G, purple), *T. ornithopodioides* (O, gold), *T. striatum* (S, black)*; T. tomentosum* (T, grey).
(B) Seed provenance origin = New Zealand (black); Spain (red); and the UK (gold).

Figure 3.5. Flavonoid concentration (log-transformed parts per million, ppm) as a factor of root biomass (log transformed grams) with coloured points and regression lines indicating (A) the five *Trifolium* species and (B) the three seed provenance origins, Spain, UK and New Zealand (NZ). The linear mixed-effects model results for the data shown in panel B can be found in Appendix F.2.

3.5 Discussion

We found multi-species evidence for some down-regulation of flavonoid richness and concentration among plants from non-native provenances when plants were grown in rhizosphere soils cultivated by conspecifics in the native range. Inter-provenance differences in flavonoid production were significant in soils from the native range and greatest in the sterilised soil treatments. Our results suggest that these non-native plants, which have been naturalised in New Zealand for many decades, have down-regulated root flavonoid production. This divergence may be in response to enemy release and dislocation from rhizosphere mutualists because differences were specifically when plants were re-exposed to microbiota from the native range. It is noteworthy that in both the New Zealand and European glasshouses, pots in the sterilised treatments did not remain sterile throughout the experiments; several of these plants hosted rhizobia and/or AMF and some soils contained visible fungal structures. However, a benefit of running separate experiments in each range is that the microbes that contaminated these sterilised pots would be microbes common to the range of interest and thus we do not consider this to be a confounding factor. In addition, the lower levels of colonisation in the sterilised pots compared to the unsterilised treatments suggests that they remained relatively biota free for most of the experimental period.

The results are also in agreement with a parallel plant-soil feedback experiment that showed rhizosphere microbes in the non-native range are generally less antagonistic to plant growth compared to microbes in the native range for these species of annual *Trifolium* (McGinn 2015). However, we found no consistent evidence for a trade-off between flavonoid richness or concentration and plant growth; nor were inter-provenance differences in flavonoid production correlated with the geographic distribution of these species. Thus, although some down-regulation of flavonoids may be occurring, it does not appear to be a factor in naturalisation success and thus the EICA hypothesis is not fully supported in this system.

3.5.1 Flavonoid production

Our prediction that non-native plants have down-regulated flavonoid production was supported by non-native plants having significantly lower flavonoid richness in both native range sterilised soils (Spain and UK origin), significantly lower richness the unsterilised UK soil, and significantly lower flavonoid concentration in sterilised Spanish soils. Our hypothesis was further supported by the lack of inter-provenance differences in the non-native range soil treatments, where flavonoid richness and concentration were similar among plants from all three seed provenances. This lack of difference in flavonoid richness in the non-native range soil treatments is evidence that the lower values seen in the native-range soil treatments were not just the result of non-native plants being naïve to novel biota. If the differences were the result of local adaptations, we would expect that plants from the

native-range would have also had lower flavonoid richness than non-native conspecifics when grown with rhizosphere microbiota from the non-native range. The inconsistency between the sterilised soil treatments (significant differences in the native range but not in the non-native range) may be interpreted as some evidence of enemy release from antagonistic rhizosphere microbiota, as sterilised glasshouse pots are colonised by common opportunists in each range. The result therefore corroborates plant-soil feedback experiments that show native-range rhizosphere microbiota are generally negative to plant growth (Willis *et al.* 2000; Mitchell & Power 2003; Torchin *et al.* 2003; Gundale *et al.* 2014) whereas microbes in the non-native range tend to have neutral or positive effects on plant growth (Inderjit & van der Putten 2010)—at least early in naturalisation (Diez *et al.* 2010; Flory & Clay 2013).

3.5.2 Daidzein: An isoflavonoid central to defence and rhizobia nodulation

Daidzein richness and concentration were lower among plants from non-native provenances compared to conspecifics in all native-range soils of both treatments, however these differences were not statistically significant (Figure 3.3; Figure 3.4). Daidzein and its precursors and derivatives are known to be intimately involved in the rhizobia mutualism (Stafford 1997; Cooper 2007; Cesco *et al.* 2010) and previous work showed that rhizobia nodulation was reduced among these non-native *Trifolium* species (Appendix D.3), therefore we had predicted a positive correlation between rhizobia nodulation and both the richness of daidzein-like compounds and their total concentration. This prediction was only partially supported. In all three unsterilised soils, daidzein *richness* was significantly and positively correlated with rhizobia nodulation, however daidzein *concentration* was only positively correlated with rhizobia nodulation in the unsterilised New Zealand soil treatment; in the two native-range soils there was a non-significant negative correlation (Appendix F.6). There are many explanations for the lack of correlation in concentration and the lack of significant differences between provenances in daidzein production. First, daidzein is a phytoalexin—a generalist defence compound produced in low levels constitutively—and differentiating flavonoids produced constitutively and in response to biotic stimuli is not possible without a truly biota-free treatment (logistically impossible in most glasshouses). Second, any potentially non-linear relationships between daidzein concentration and nodulation would have been undetectable with our coarse-scale method (0–3 count) of nodulation scoring. Third, the multi-functional nature of daidzein (mediator of rhizobia interactions, defence against rhizosphere pests and pathogens) make it impossible to detangle the cause of the compound's production without testing flavonoid production in the presence of specific fractions of the soil microbiota. In addition, while small amounts of many flavonoids serve as chemo-attractants to beneficial microbes, high amounts of the same flavonoids will serve to repel or even kill microbes that lack appropriate chemical cues, and these threshold values have yet to be characterised for most plant species (Weston & Mathesius 2013). Thus, the

beneficial effects of lost enemies and the negative effects of being dislocated from mutualists may cancel each other out in the case of daidzein production.

3.5.3 Secondary metabolites in the context of invasion

Plants equipped with a diverse arsenal of compounds may be superior at combating stress at the early stages of naturalisation (Lockwood *et al.* 2007). On the other hand, once plants establish self-sustaining and spreading populations in the non-native range, trading superfluous compounds in favour of fitness could be a mechanism for increased invasibility (Keane & Crawley 2002; Joshi & Vrieling 2005). Thus, high metabolite diversity may present an advantage to colonisers, but have diminishing returns as a population establishes and the local soil biota equilibrates (Levine *et al.* 2006; Kardol *et al.* 2007; Gundale *et al.* 2014). Because rhizosphere enemies can catch up with plant invaders (Diez *et al.* 2010), losing the ability to produce some defence compounds may become a limitation to further invasion over time. However, if non-native plants have (or are selected for) phenotypic plasticity in this trait, they may be able to down-regulate constitutive secondary metabolite production (thus conserving energy if antagonistic interactions are less frequent or intense), while maintaining the ability to produce defences or mutualist-associated compounds when needed. For example, non-native *Alliaria petiolata* (Brassicaceae) have lower constitutive production of glucosinates, but these compounds have higher inducibility (Cipollini *et al.* 2005), so that plants are still well protected from generalists when they are attacked. Future studies of post-naturalisation biochemical shifts should incorporate more secondary metabolites from above- and belowground as well as acquire long-term data that span the different stages of naturalisation to detect shifts in biochemistry—both constitutive and metabolites produced in response to novel biota in the non-native range.

3.5.4 Conclusions

Post-naturalisation differences in plant chemistry provide invaluable evidence about how non-native plants respond to new rhizosphere communities and may adapt to their new environments. In our study, we found evidence that plants from non-native provenances have significantly down-regulated production of root flavonoids. However, we found no evidence that the loss of these flavonoids equated to a performance trade-off in line with the predictions of EICA, nor did the differences in flavonoid production between provenances have a positive correlation with the geographic distributions of each species. Thus, altered flavonoid profiles are probably not a factor in the naturalisation success of these *Trifolium* in New Zealand.

Chapter 4

No evidence for increased competitive ability among non-native *Trifolium*

4.1 Abstract

Rhizosphere microbial communities are intrinsically different among plant communities and geographic locations, yet few studies in plant-invasion ecology have grown plants in rhizosphere soils from the native and non-native ranges when testing for post-naturalisation differences in performance or competitive ability. The evolution of increased competitive ability (EICA) hypothesis suggests that escape from pests, pathogens and herbivores can result in selection for loss of defensive traits and increased competitive ability; similarly, dislocation from mutualists can trigger reallocations to compensate for lost benefits. Here, we test the EICA hypothesis in the context of rhizosphere microbial communities from each range—and we expand the EICA framework by taking a whole-soil approach that includes both antagonists and mutualists. We hypothesised that, relative to native provenances, plants from non-native provenances will have: i) greater growth rates and greater competitive ability when grown in non-native range soil due to escape from antagonistic rhizosphere microbiota and reallocation of resources from defences or missing mutualisms to growth; and ii) lower growth rates and less competitive ability when reintroduced to rhizosphere microbiota from their native range, due to losing defence- and mutualist-related abilities post-naturalisation. For three widely naturalised, non-agricultural *Trifolium* species, we collected seed and rhizosphere soil from native (Spain and the UK) and non-native (New Zealand) provenances and grew plants from each provenance singly and in competition with a conspecific from a different provenance to compare the relative competition intensity (RCI) of plants from each provenance in the presence of rhizosphere microbiota from each range. In contrast to expectations, non-native plants were not more competitive than native conspecifics in non-native range soil. In addition, although in native-range soils, plants from non-native provenances of two species grew slower than native provenances, as predicted by EICA, these provenances were no less competitive in native-range soils. Our results revealed a surprising finding: the slower growth of plants from non-native provenances in native-range soils did not translate to lower competitive ability in native-range soils, as has been assumed under previous tests of EICA. The lack of correlation between measures of growth and measures of competitive ability highlights the importance of performing direct tests of plant competition in invaded systems—as measures of growth are not always an appropriate surrogate for determining competitive ability.

4.2 Introduction

A central goal in ecology is to identify the mechanisms underlying the success of invasive plants (Keane & Crawley 2002). One of the most prominent explanations is the evolution of increased competitive ability (EICA) hypothesis, which proposes: (i) that non-native plants benefit from escaping their specialist enemies from the native range (Liu & Stiling 2006), (ii) that enemy escape can lead to selection for genotypes with reduced investment in costly defence traits (Doorduin & Vrieling 2011), and (iii) that these shifts in energetic investment can favour adaptations toward greater competitive ability (Blossey & Nötzold 1995). Despite a large body of evidence supporting post-naturalisation adaptations (Prentis *et al.* 2008), few tests find support for the full set of EICA predictions (Bossdorf *et al.* 2005). For example, plants in the non-native range may be larger than conspecifics from the native range, without an apparent loss of defences (Alba *et al.* 2011); non-native plants may be both larger and better defended than they were in the native range (Ridenour *et al.* 2008; Abhilasha & Joshi 2009; Caño *et al.* 2009); and some studies contradict EICA entirely because the non-native plant shows no evidence of increased performance—being smaller (Daehler & Strong 1997) or having reduced competitive ability (van Kleunen and Schmid 2003; Bossdorf et al. 2004) relative to native conspecifics.

One explanation for the equivocal findings of EICA is that studies have used a variety of measures to quantify differences in performance between plants from native and non-native provenances, instead of using a standard, direct test of competitive ability. A recent meta-analysis of the EICA literature revealed that of 58 EICA studies only 10 measured competitive ability directly and in all cases competitive ability of native and non-native provenances was assessed relative to a heterospecific (Felker-Quinn et al. 2013). Using a heterospecific as a "phytometer" against which to measure the competitive ability of plants from native and non-native provenances may confound competitive effects with species-specific interactive effects (Maron et al. 2004), such as allelopathy (Ridenour *et al.* 2008; Qin *et al.* 2013), differences in root architecture (Rubio 2001), and differences in how species or genotypes culture soil biota or affect nutrient dynamics (van der Putten et al. 2007; Ridenour et al. 2008). For example, nitrogen availability has been found to be negatively correlated with competition intensity (Wilson & Tilman 1993), suggesting that nitrogen-fixing species may actually increase the competitiveness of neighbouring species by supplying a limiting nutrient. Because nitrogen-fixers (i.e. Fabaceae) are among the world's most widespread invasive plants (ISSC 2015), and similar facultative traits likely exist among other taxa, tests of the competitive ability should be standardised to avoid confounding effects.

Another explanation for the lack of consistent results among EICA tests is that nearly all experimental designs have failed to integrate the rhizosphere microbial communities from an invasive plant's

native and non-native ranges, which are inherently different due to the dispersal limitations of most microbes (Pringle *et al.* 2009; Litchman 2010; Tedersoo *et al.* 2014). Plant-soil feedback experiments and studies of enemy release show that the plant-growth effects of rhizosphere communities in the native versus non-native ranges differ greatly (Bever *et al.* 1997; Reinhart *et al.* 2003, 2010; Callaway *et al.* 2004; Engelkes *et al.* 2008; Inderjit & van der Putten 2010; Andonian & Hierro 2011; Gundale *et al.* 2014). These microbiota play key roles in plant community composition (Coats & Rumpho 2014) and competition (van der Putten & Peters 1997). For example, arbuscular mycorrhizal fungi provide great competitive advantage by exponentially increasing root surface area and therefore acquisition of both water and nutrients (Sabais *et al.* 2012), while nitrogen-fixing rhizobia directly provide a limiting nutrient (Richardson *et al.* 2000a), and other root endophytes induce systemic resistance, making plants better able to combat future enemies and environmental stress (Pieterse *et al.* 2014). The absence of rhizosphere antagonists is a form of enemy release that fits well in the EICA framework (Maron *et al.* 2014) and has been well supported empirically (Diez *et al.* 2010; Andonian *et al.* 2011; Callaway *et al.* 2011), while more studies are showing that mutualists (or the lack thereof) can also play a role in selection (Seifert *et al.* 2009; Porter *et al.* 2011). However, investigations of post-naturalisation plant performance in the presence of rhizosphere communities from each range have only been tested partially; for example, Volin *et al.* (2010) analysed performance of the fern *Lygodium microphyllum* (Lygodiaceae) in soils from each range, but only in the absence of competition.

Here, we present a multi-species test using the EICA framework to compare both the performance and the competitive ability of plants from native and non-native provenances when grown in rhizosphere soil cultivated *in situ* by conspecifics in the native and the non-native ranges. We define competition as the reduction in growth associated with acquiring resources in a shared environment (Casper & Jackson 1997; McKenney *et al.* 2007). By growing plants of the same species from different provenances in direct competition with each other, we remove the confounding effects inherent in standard comparisons of interspecific interactions (Bossdorf *et al.* 2004; Beaton *et al.* 2011; Liao *et al.* 2013), allowing us to directly test for evidence of post-naturalisation differences in competitive ability. We carried out this study using three species of *Trifolium* native to Europe that have naturalised widely in New Zealand. Because we wished to test for post-naturalisation differences in competitive ability, we selected a system where plants of non-native provenances are likely to face intense competition. All selected species are naturalised in a variety of environments, including human-disturbed, ruderal locales where competition with grasses and opportunistic forbs is ubiquitous (Maxwell 2013). Moreover, the *Trifolium* species chosen for this study co-occur throughout the New Zealand-naturalised range in a variety of habitats with several agricultural congeners, including competitive perennials *T. repens* and *T. pratense* (Boswell *et al.* 2003), which

suggests that these three species are likely to be in competitive environments in much of their naturalised range. Moreover, our test matched conspecific plants, and closely related taxa tend to evoke more intense competitive scenarios than heterospecifics (Gerlach & Rice 2003). Thus, we predict competition to be a potential mechanism of selection in New Zealand.

We tested two predictions: (1) If non-native plants are dislocated from co-evolved biota and reallocate resources (e.g. defences against antagonists and chemical metabolites that stimulate mutualisms) toward growth, then plants from non-native provenances will grow faster and have greater competitive ability than native provenances when grown together in non-native range soil; and (2) When reintroduced to native-range soil rhizosphere communities, plants from the non-native provenance, having reallocated resources away from defences and lost mutualisms, will grow slower and be less competitive than native conspecifics.

4.3 Methods

4.3.1 Study species

To test our predictions, we used three species of *Trifolium* (*T. arvense, T. campestre* and *T. striatum*) native to Europe but accidently introduced and naturalised in New Zealand. We restricted our study to non-agricultural species so that any provenance differences were not the result of selective breeding. Selected species have traits amenable to rapid adaptation in a new environment: they are annuals that spread by seed, they are predominantly out-crossers and they have been successful in a wide range of habitats following their introduction to many regions worldwide (Boswell *et al.* 2003; Atwood & Meyerson 2011). All three species naturalised in New Zealand before 1876 and have had more than 130 years to adapt to local conditions (Whitney and Gabler 2008; Willis et al. 2000). See Table 4.1 for detailed species information.

4.3.2 Rhizosphere collection

To compare how plants from native and non-native provenances performed in association with belowground soil biota from both the native and non-native ranges, we inoculated glasshouse pots with rhizosphere soil cultivated *in situ* by plants of each of the three *Trifolium* species in each range. In the non-native range, we collected soil from five sites for each species from Banks Peninsula, Canterbury, New Zealand. All three species co-occur here in high abundance (Appendix A.3), and the region comprises a variety of habitats broadly representative of where these species have naturalised on the South Island (Boswell *et al.* 2003).

Table 4.1. Summary information for the three species of *Trifolium* used in this study. Rhizobia nodule scoring details can be found in Appendix C.1.

			Distribution		Performance in glasshouse trials		
Species	**Years naturalised in New Zealand***	**Date naturalised in New Zealand***	**Non-native range ‡***	**Native range ¶***	**Rhizobia nodule score (Mean ± S.E.)**	**Dry-weight biomass (Mean g ± S.E.)**	**Seed size (Mean mg ± S.E.)**
T. arvense	138	1876	83	26.6	1.6 ± 0.1	0.6 ± 0.1	0.38 ± 0.01
T. campestre	147	1867	46	20.1	1.8 ± 0.1	0.8 ± 0.1	0.30 ± 0.00
T. striatum	138	1876	44	10.1	1.6 ± 0.1	2.2 ± 0.1	1.83 ± 0.07

‡ Number of 10 x 10 km NZMS260 grids occupied by at least one population; Gravuer 2004
¶ Area estimate (x 10^{12} km^2); Gravuer 2004
* Data from Gravuer 2004

In the native range, we collected soil from five sites for each species in each of two countries, the southern United Kingdom (UK) and northern Spain. Ideally tests investigating post-introduction adaptation compare performance in soils from the non-native range with performance in soils from that part of the native range from which the species were introduced (Gundale *et al.* 2014). The origin of the founding populations for these accidentally introduced clovers is unknown (Gravuer 2004), but many of New Zealand's agricultural clovers were imported from the UK making it a likely source location and an appropriate native-range comparison. We also included provenances and soils from northern Spain, as all three species are common in this region and the latitude closely matches our sampling locations in the non-native range, which may minimise any performance differences associated with latitudinal clines (Colautti et al. 2009).

The five soil collection sites in each country were located between 1 km and 221 km apart, to encompass a range of soil and land-use types. At all sites, the species of interest co-occurred with congeners, particularly the agricultural species *T. repens.* In six cases, we collected soil for two study species at the same site (Appendix A). At each site, we collected approximately 100 mL of rhizosphere soil from directly beneath 10 plants located at least 1 m apart. Equipment was sterilised between sites to keep replicates independent. Soil from each site was air-dried (Reinhart *et al.* 2003), bulked and sieved to 4 mm. We also removed all visible macrobiota and roots before storing the soils in sealed bags in cool storage rooms (16-22°C).

4.3.3 Seed collection

We sourced seed of each species from one site in the non-native range (NZ) and one site in each country in the native range (Spain and UK) (Appendix A) Seed was hand-collected from a minimum of 12 plants, homogenized, cleaned and tested for viability prior to the experiments. For *T. arvense* in the UK, seed collected from wild populations was sourced from Herbiseed, a UK germplasm centre, because plants in the field were not setting seed at the time we collected soil. Although seed from any single population will not capture the genetic diversity in a given range, in this study, species is the intended level of replication, as each *Trifolium* species has its own suite of optimal rhizobia mutualists and rhizosphere antagonists, and thus each species forms an independent comparison between the performance of plants from the native and non-native provenances. In addition, we expected differences in growth rate and competitive ability between native and non-native provenances to be stronger than the variation among populations within each range (Leger and Rice 2003; Buschmann et al. 2005; Erfmeier and Bruelheide 2005; Blumenthal and Hufbauer 2007). Seeds were sterilized in a 10% solution of bleach for 2 min, rinsed thoroughly in DI water and scarified gently with a scalpel to break the hard seed coat. Seeds were germinated on sterile glass beads

under species-specific temperature and day-length requirements in a germination cabinet (Appendix B.1).

4.3.4 Glasshouse experiments

To compare the performance of plants from native and non-native provenances in the presence of rhizosphere microbiota from each range, we conducted two separate glasshouse experiments. The test with non-native-range soil was carried out at Lincoln University in Canterbury, New Zealand, in Southern Hemisphere summer 2013. This experiment tested the prediction that the growth rate and competitive ability of plants from non-native provenances would be greater than those of plants from native provenances when grown in soil from the non-native range as a result of being exposed to different rhizosphere communities, including mutualists and antagonists, and subsequently diverting resources (e.g. defences or mutualist-enhancing metabolites) into growth. We created two treatments for each species in each soil: single-plant pots and paired-plant pots. In the single-plant treatments, a plant from each provenance was grown singly with an inoculum of rhizosphere soil from one of the five soil collection sites, replicated twice to give 90 single pots (3 species x 3 provenances x 5 soil sites x 2 replicates). In the paired-plant pots, a plant from the non-native provenance was grown in competition with a plant from one of the native provenances (either UK or Spain) with an inoculum of rhizosphere soil from one of the five soil collection sites replicated twice, giving 60 paired-pots (3 species x 2 native provenances x 5 soil sites x 2 replicates).

The experiment with soils from the native range was conducted at The Netherlands Institute of Ecology in Wageningen, The Netherlands, in Northern Hemisphere summer 2013. This experiment tested the hypothesis that plants from non-native provenances would grow more slowly and be less competitive than plants from the native provenances when exposed to native-range soil biota as a result of these plants having shifted resources (e.g. away from defences and mutualisms) in the non-native range. Pots were inoculated with unsterilised soil from either the UK or Spain.

The sandy background soils that formed the bulk of each pot were sterilised by two successive rounds of autoclaving (20 min at 121° C) in New Zealand and by gamma irradiation (>25 kGray) in The Netherlands. No fertilizers or soil amendments were used in either glasshouse, as sterilised background soil provides sufficient nutrients. Autoclaving did not appear to induce chemical changes damaging to plant growth and although this method of sterilising soil can alter soil structure, I suspect these differences to be minimal because we used sandy soils with only about 2-3% organic matter. Further, the organic matter and total nitrogen content of background soils were comparable between the two glasshouses (see Appendix B2). A 10% (v/v) inoculum of unsterilised rhizosphere soil was mixed into the background soil in each pot to provide ample biota without strongly influencing abiotic properties, such as pH, nutrients and organic matter (van der Putten *et al.* 2007;

Maron *et al.* 2014). Using a fraction instead of the whole soil also serves to standardise the effect of nutrient flushes from the sterilised inocula. Seedlings were transplanted into the treatment pots soon after all replicates had their first true leaves. Only seedlings that died within the first week were replaced. At the end of the experiments, there were 187 replicates from the single-plant pot treatment (60 in Spanish soil, 54 in the UK soil and 73 in New Zealand soil) and 98 replicates from the paired-plant pot treatment (30 in Spanish soil, 26 in UK soil and 42 in New Zealand soil). All mortality occurred in the first two weeks of the experiments and was not attributable to competition effects.

Pots were assigned to a random location in the glasshouses and moved every two weeks. Single-plant pots and paired-plant pots were watered to a species-standardised weight on a weekly or twice-weekly basis as needed. Plants of the same species were harvested on the same day after approximately three months when plants began forming flower buds, indicating an energetic switch from growth to reproduction, and it was clear that plants were nearing pot capacity. In preliminary experiments with single-plant pots all three selected species grew to pot capacity in about three months, suggesting this was a suitable pot volume and growing period to ensure competitive conditions. Roots were washed gently and colonization by the nitrogen-fixing symbiont *Rhizobium leguminosarum* bv. *trifolii* was scored on a 0-3 scale following a modified protocol from Corbin et al. (1977) that takes into account the number, size, location, colour and effectiveness of nodules (Appendix C.1). Roots and shoots were separated and oven-dried at 65° C. We used growth rate (dry biomass / number of glasshouse growing days) to standardize comparisons among species.

4.3.5 Analysis of EICA competition studies

To inform our study design and to provide context for our results, we compiled a summary table of the EICA competition studies to date that tested both growth (either overall size or growth rate) and competitive ability (Table 4.2). We first performed a Web of Science literature search using the key words “EICA” and “competition.” Our search results yielded 130 studies and these matched well with two recent meta-analyses, so we are confident that our search was comprehensive (Bossdorf et al. 2005; Felker-Quinn et al. 2013). From this list, the criteria for inclusion in the table was that studies had to: (1) use original, experimental data to test the EICA hypothesis; (2) grow plants sourced from both the native and non-native ranges in a common environment (either a glasshouse or a field plot); (3) perform a growth- or size-based performance test in the absence of competition and (4) perform either an intra-specific or inter-specific test of competition.

4.3.6 Statistical analyses

We first compared the growth rates of singly grown plants from each of the three provenances using separate linear mixed-effects models for each species in soil from each provenance (New Zealand,

Spain and UK). Each analysis was run separately in each native range soil allowing us to test for inter-provenance differences independently in each country. Growth rate was log-transformed to meet parametric assumptions. We accounted for potential non-independence due to site-specific effects by including the site from which soil was collected as a random effect in the models. Because *Trifolium* growth can be dependent on the degree of *Rhizobium* association, and differential nodulation with *Rhizobium* rather than shifts in resource allocation could explain inter-provenance differences in growth rates (Appendix H.2), we included nodulation score as a fixed effect in our model (Appendix H.1). Doing this provides a measure of comparative growth rate having accounted for the effect of nodulation on growth. To test for a significant difference in growth rate among plants from different provenances grown in the same soil, we ran an analysis of variance on the difference between the model that included seed provenance as a fixed effect and the one with seed provenance removed.

To compare the competitive ability of plants from native and non-native seed provenances grown in soil from each provenance, we used the relative competition intensity (RCI) index, calculated as:

$RCI_{A(B)} = \frac{GR_A - GR_{A(B)}}{GR_A}$ where GR_A is the growth rate of a plant from provenance A when grown alone and GR_{AB} is the growth rate of a plant from provenance A when grown in competition with a plant from provenance B. Higher values of RCI (up to a maximum of 1) indicate a stronger competitive effect of the provenance B plant on the provenance A plant. A value of zero indicates there was no competitive effect; values less than 0 indicate a facilitative effect (i.e. a plant from provenance A grew better when grown with a plant from provenance B). RCI and similar measures of competition intensity have been widely used in studies of community ecology and this allows us to compare our results to the few tests of EICA that have included a competition index (Liao et al. 2013; Oduor et al. 2013; Qin et al. 2013; Vilà and Weiner 2004).

For each species, we calculated RCI values by first fitting a linear mixed-effects model to the (log-transformed) growth rates of plants from single-plant and paired-plant treatments in each soil type (New Zealand, Spain and UK), including the site from which soil was collected as a random effect. We fitted this model without an intercept and with a variable that coded for the seed provenance (for single-plant pots) or seed-provenance combination (for paired-plant pots) as a fixed effect. As with the growth-rate model, we included as a fixed effect the plant's *Rhizobium* nodulation score to remove its effect. We extracted from this model the mean growth rate and associated uncertainty for each seed provenance and seed-provenance combination having accounted for site effects. We used these mean growth rates and their uncertainties to calculate the RCI indices (Appendix H).

To allow the uncertainties associated with the estimates of mean growth rate to propagate into the RCI index we used a simulation approach, extracting the variance-covariance matrix for the fixed effects from the fitted models. These variance-covariance matrices provide estimates of the mean growth rate of single and paired plants, along with their variances and co-variances. We then drew 100,000 random values from the normal distributions defined by these variance-covariance matrices to obtain estimates of the mean growth rates and used these values to calculate 100,000 values for each RCI index, from which we obtained the means and 95% confidence intervals. For each species in each soil type (New Zealand, Spain and UK) we calculated two RCI indices for each native-non-native provenance pair. In NZ soil, for example, we calculated $RCI_{NZ\text{-}SP}$, which measures the competition intensity experienced by the non-native (New Zealand) provenance when grown with the native (Spanish) provenance, and $RCI_{SP\text{-}NZ}$, which measures the competition intensity experienced by the Spanish provenance when grown with a plant from the New Zealand provenance.

To compare the competitive ability of native and non-native provenances of each species in each soil, we subtracted the RCI index of the native provenance (e.g. $RCI_{SP\text{-}NZ}$) from the RCI index of the non-native provenance (e.g. $RCI_{NZ\text{-}SP}$) for each of the 100,000 simulated values. The resulting means and 95% confidence intervals measure the difference in competitive ability between native and non-native provenances in the same soil, and the associated uncertainty. A value of zero would indicate no difference in competitive ability; values greater than zero indicate the native provenance was more competitive; and negative values indicate the non-native provenance was more competitive. We assessed the significance of these differences by whether the 95% confidence intervals overlapped zero.

Lastly, to test whether differences in growth rate translate to differences in competitive ability, we tested for a correlation between the growth-rate differences and the RCI value differences between native and non-native plants across all species and soils. The experimental design and statistical analyses were set up to allow us to identify and quantify differences between provenances in the factors of interest—including differences in the opposite direction predicted. All statistical analyses were performed using R (ver. 3.0.2) (R Development Core Team 2013). Model scripts can be found in Appendix H.1. Linear mixed-effects models were fit using the lmer function, which uses restricted maximum likelihood, in the R package “arm” ver. 1.6.10 (Gelman *et al.* 2014).

4.4 Results

4.4.1 Growth in the absence of competition

When grown in soils from the non-native range, there was no clear difference in growth rate between plants from the native and non-native provenances for all three *Trifolium* species (Figure

4.1). This is contrary to what we would expect if plants from non-native provenances had diverted resources post-naturalisation that increased their competitive ability. In native-range soils from Spain and the UK, however, plants from non-native provenances of *T. arvense* and *T. striatum* on average grew slower than plants from each of the native provenances, as expected if these plants had during invasion lost characteristics relevant to interactions with co-evolved biota (e.g. defences or mutualist-stimulants) and were then re-exposed (e.g. to antagonists or mutualists) (Figure 4.1). For these two species, the inter-provenance differences in growth were often substantial: plants of *T. arvense* from non-native provenances grew about half as fast on average as the native provenances in both Spanish soil ($F_{1,20}$ = 76.34; P < 0.001) and UK soil ($F_{1,15}$ = 6.50; P = 0.03), while *T. striatum* plants from non-native populations grew 32% slower than natives in UK soil ($F_{1,19}$ = 9.77; P = 0.04). Non-native *T. striatum* also grew 20% slower than natives in Spanish soil, but this difference was not significant ($F_{1,20}$ = 14.39; P < 0.93) In contrast, *T. campestre* showed the opposite pattern, with plants from the non-native provenance growing 36% faster than UK plants in UK soil ($F_{1,20}$ = 19.44; P < 0.001). Non-native *T. campestre* also grew 12% faster than Spanish plants in Spanish soil, but this difference was not significant ($F_{1,20}$ = 3.08; P = 0.90).

4.4.2 Competitive ability

Competition significantly reduced plant growth rates, with plants in paired-plant treatments growing slower than plants grown in single-plant pots by an average of 35% ($F_{1,384}$ = 49.13; P < 0.001), confirming that our paired-plant treatments had created competitive conditions. However, the results of the competition experiments do not support the EICA hypothesis.

Figure 4.2 shows the RCI indices for provenances grown in soil from each country; with a few exceptions, native and non-native provenances have similar competitive ability. This is confirmed in Figure 4.3, which plots the difference in RCI between provenances when plants are grown in the soil from each country. In non-native soils, non-native *T. striatum* were slightly more competitive than native conspecifics from the Spanish provenance, consistent with EICA, but for the other two species there was either no difference between provenances or, in the case of *T. arvense*, the native UK plants were slightly more competitive than those from the non-native provenance (Figure 4.3).

In native-range soils, the only clear difference was that non-native provenances of *T. striatum* were more competitive than native range provenances—the opposite of what is predicted based on EICA. Overall, growth rate of a seed provenance in the single-plant trials was not indicative of its competitive ability in the paired trials. Rather, there was a significant negative correlation between the magnitude of the difference in growth rate between provenances and the magnitude of the differences in RCI values between provenances (Pearson's correlation = -0.69; P = 0.01), so that

although sometimes the growth-rate differences between plants from native and non-native provenances were substantial, they did not correspond to a substantial difference in competitive ability, as would be expected.

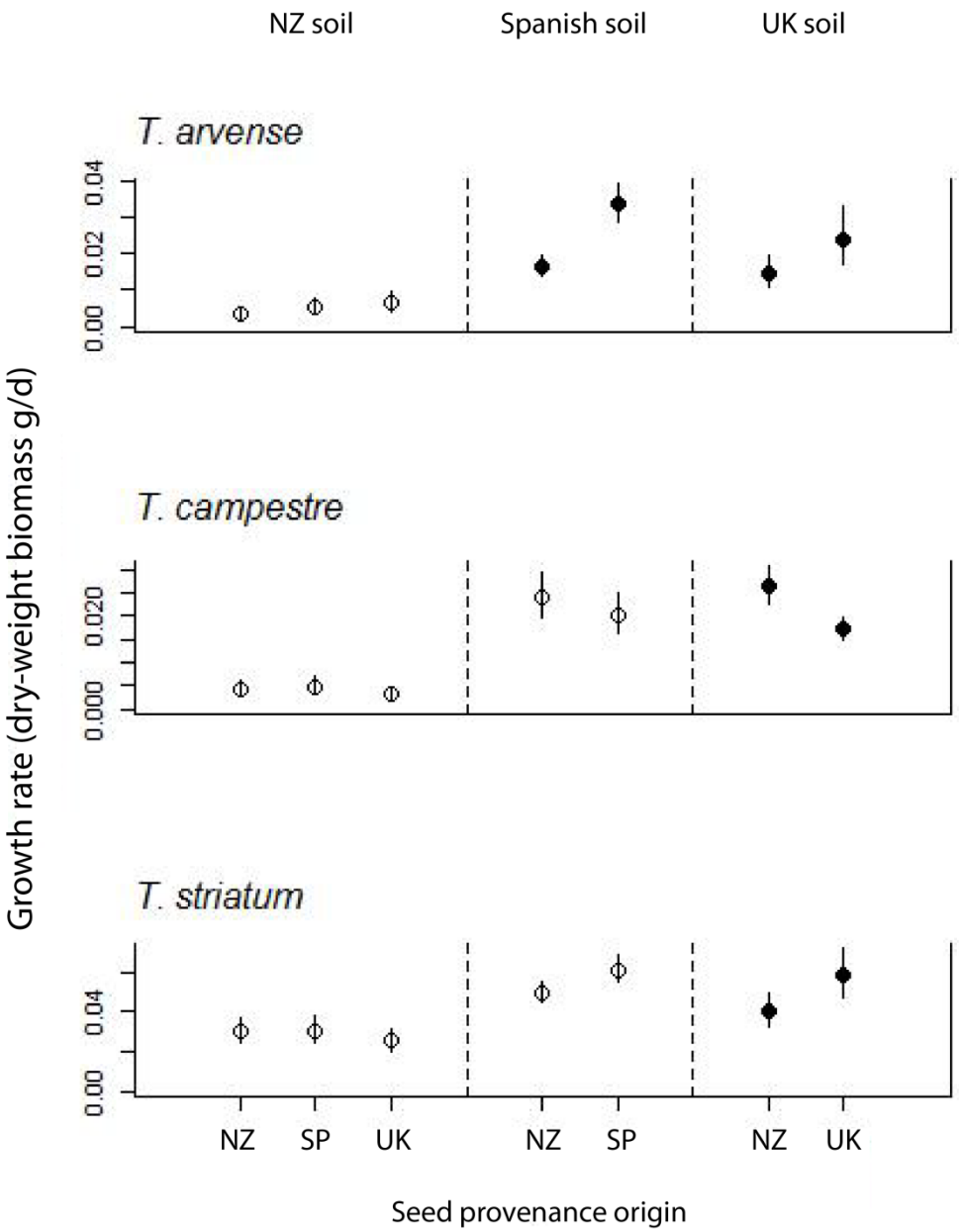


Figure 4.1. Model-adjusted growth rates of plants from the non-native (New Zealand, NZ) and native (Spanish, SP, and UK) seed provenances of the three *Trifolium* species grown singly in pots inoculated with rhizosphere soil cultured by conspecifics in New Zealand, Spain and the UK. Error bars are 95% confidence intervals. Filled circles represent inter-provenance differences that are statistically significant (P < 0.05).

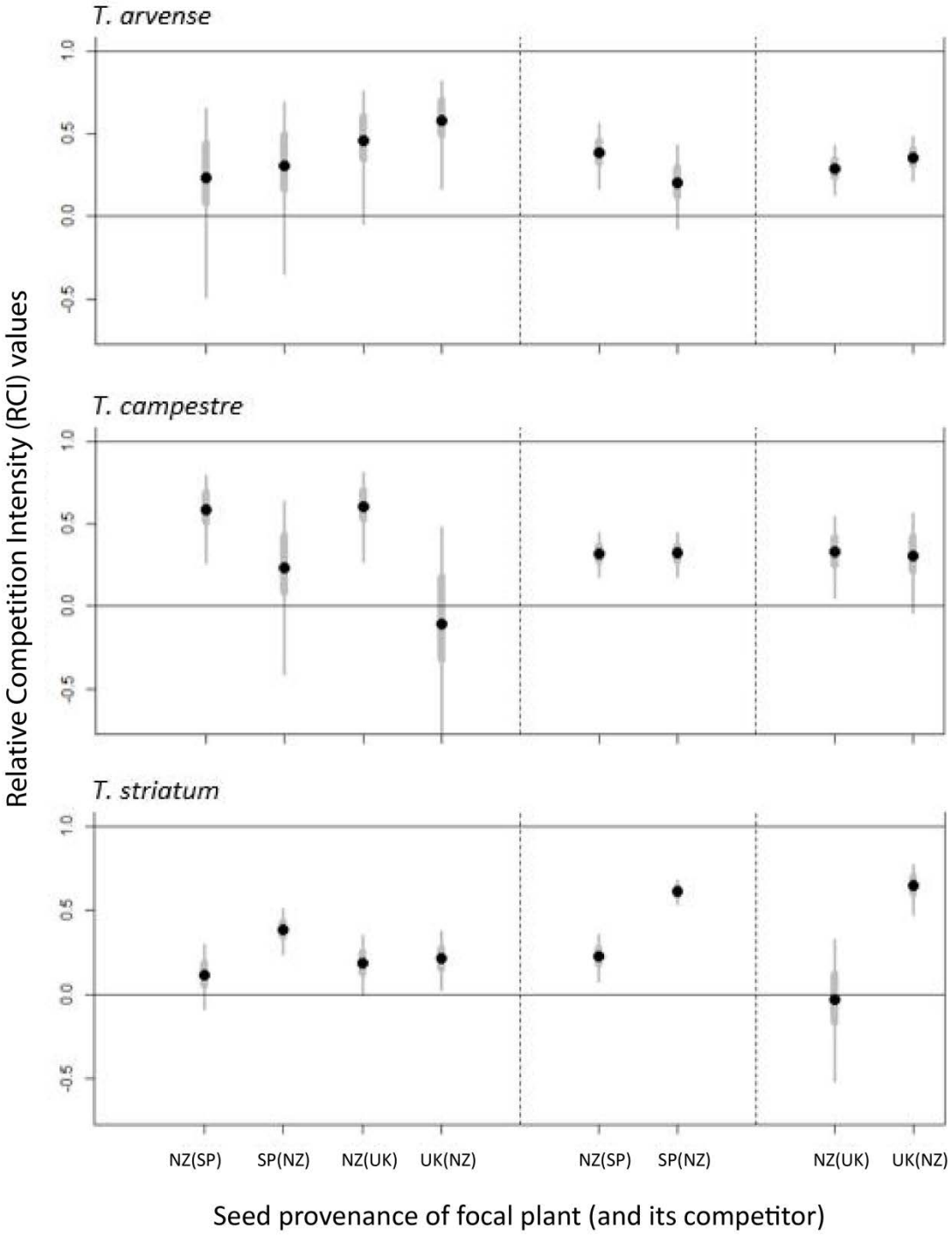


Figure 4.2. Relative competition intensity (RCI) indices for plants of from the non-native provenance (New Zealand, NZ) and the native-range provenances (Spain, SP, and the UK) for three *Trifolium* species grown in pots inoculated with soil from each location. The RCI index is calculated as follows: $RCI_{A-B} = (GR_A - GR_{AB}) / GR_A$ where GR_A is the growth rate of provenance A grown alone and GR_{AB} is the growth rate of provenance A grown in competition with provenance B. Higher RCI values (up to a maximum of 1) indicate a stronger competitive effect of provenance B on provenance A; zero indicates no effect of competition. Error bars are 50% (thick grey bars) and 95% (thin bars) confidence intervals.

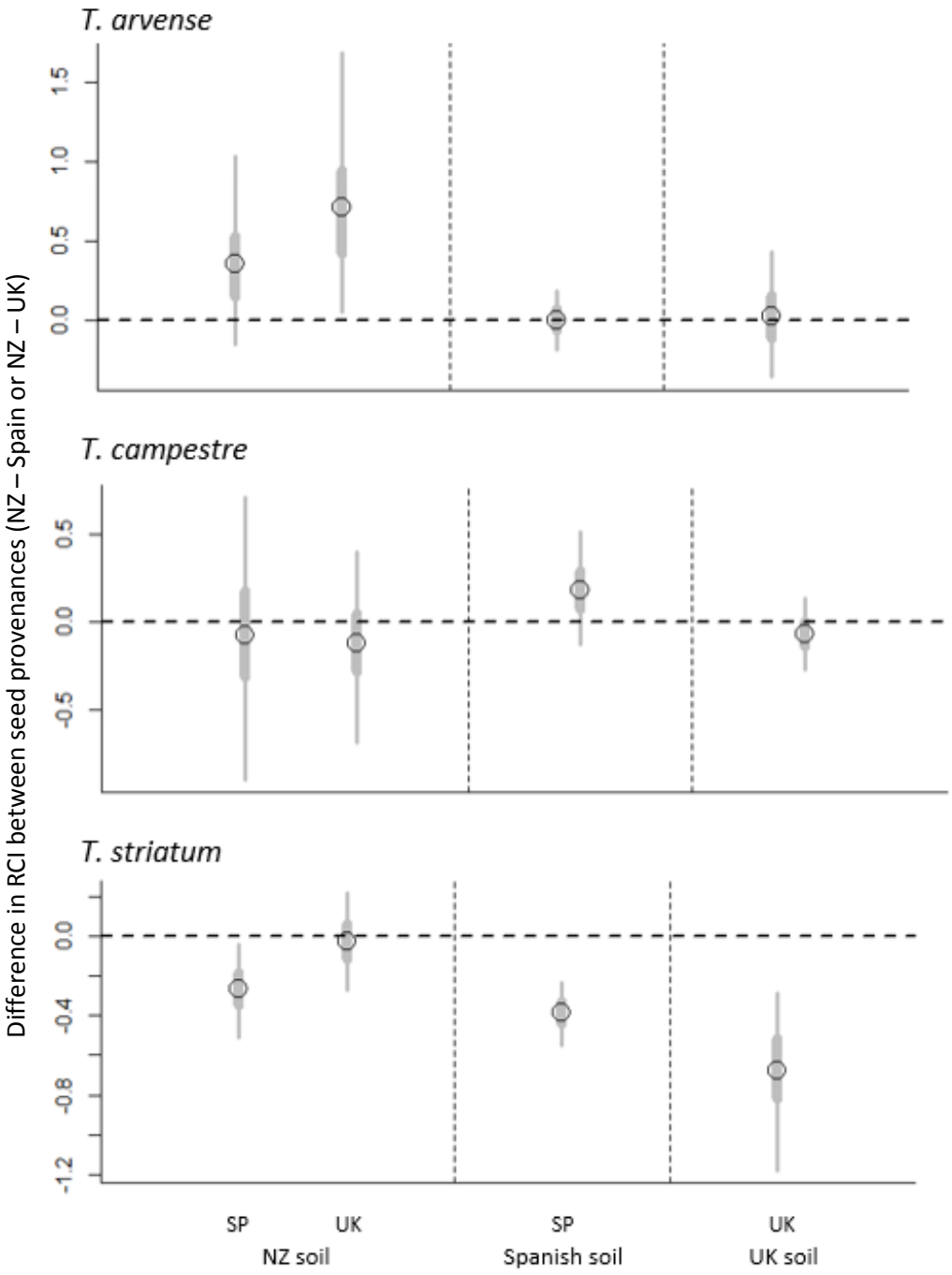


Figure 4.3. The difference in relative competition intensity (RCI) values between plants from the non-native provenance (New Zealand, NZ) and each native-range provenance (Spain, SP, or the United Kingdom, UK) of the three *Trifolium* species grown in pots inoculated with soil from each provenance. A value < 0 indicates the non-native population was more competitive than the native population. Error bars are 50% (thick grey bars) and 95% (thin bars) confidence intervals.

Table 4.2. Summary table of EICA studies in the literature that included measures of both growth performance and competition. G indicates whether the non-native provenance grew larger/faster (+), smaller/slower (-) or the same (o) as the native provenance. C indicates whether the competitive ability of the non-native provenance was greater (+), less than (-) or the same (o) as the native provenance. Where multiple tests were done, both results are included. Studies were retrieved using a Web of Science search on April 11, 2014, and included if they met the criteria outlined in Section 4.3.5.

Reference	Species	G	C	Soil types used	Type of competition	Competition index used
Blair & Wolfe 2004	*Silene latifolia*	(+)	(o)?	Potting soil	inter	NA
Blumenthal & Hufbauer 2007	14 species	(+)	(o)	Potting soil	inter	NA
Bossdorf *et al.* 2004	*Alliaria petiolata*	(-)	(-)	Potting soil	intra	NA
Graebner *et al.* 2012	*Centaurea solstitialis*	(+)	(+)	50% naturalised-range soil	inter	NA
He *et al.* 2009	*Centaurea macrophylla*	(+)	(o)(-)	25% naturalised-range soil	inter+intra	NA
Leger & Rice 2003	*California poppy*	(+)	(o)	Potting soil; native-range field plots	inter	NA
Liao *et al.* 2013	*Chromolaena odorata*	(-)	(+)(o)	70% naturalised-range soil	intra	RCI = p-s/ s
McKenney *et al.* 2007	*Lepidium draba*	(o)	(o)	Potting soil	inter	NA
Oduor *et al.* 2013	*Brassica nigra*	(o)	(o)	Native-range field plot	inter	RCII = p-s / p+s
Qin *et al.* 2013	*Chromolaena odorata*	(-)(o)	(+)(o)	70% naturalised-range soil; native-range field plot	inter	RCI = p-s /p
Ridenour *et al.* 2008	*Centaurea macrophylla*	(+)	(+)	20% naturalised-range soil	inter	NA
Rogers & Siemann 2004	*Sapium saperiferum*	(+)	(o)	Potting soil	inter	interspecific
Vilà *et al.* 2003	*Hypericum perforatum*	(o)	(o)	Potting soil	inter	RCI = p-s/ s
Present study	3 *Trifolium* spp.	(+)	(o)	10% (v/v) non-native- and native-range soils	intra	RCI = p-s/ s

4.5 Discussion

We found no consistent evidence for increased competitive ability among three widely naturalised *Trifolium* species in New Zealand. Of the 12 comparisons of competitive ability between plants from native (Spain and the UK) and non-native (New Zealand) provenances (Figure 4.3) only one result was in the direction predicted; the remainder showed no difference in competitive ability between provenances or a difference in the direction opposite to that predicted. This result was unexpected given the substantially lower growth rates among plants from the non-native provenance of two species when grown singly in rhizosphere soil from the native range (Figure 4.1). Our results revealed a surprising finding: the slower growth of plants from non-native provenances in native soils did not translate to lower competitive ability, as has been assumed under many previous tests of the EICA hypothesis (Felker-Quinn *et al.* 2013).

4.5.1 Competition in the context of invasion

We suggest three potential explanations for the unexpected lack of increased competitive ability in this system. (i) As successful naturalisers throughout much of their native ranges, *Trifolium* species may already be excellent competitors, with little ability or need to adapt this trait further. In their native range, *Trifolium* typically co-occur with congeners (Gilbert and Taylor 2001) and in New Zealand, 16 non-agricultural species of *Trifolium* have naturalised widely (Gravuer 2004) and typically co-occur with competitive forbs and grasses as well as perennial *Trifolium* (Maxwell 2013, Boswell et al. 2003). Although during the course of field work in both ranges it was observed that all three species co-occurred in close proximity with congeners and heterospecifics, we assessed neither the ability of individual plants to acquire resources nor total resource levels. (ii) Resources in the non-native range may be sufficient to not evoke competitive adaptation. (iii) In general, competition may be a less important strategy among legumes that host nitrogen-fixing bacteria, because plants are less likely to be nutrient-limited and they can colonise low-nutrient soils where competition is less intense, and these species tend to be ruderal in New Zealand (Webb, Sykes & Garnock-Jones 1988). A final consideration is that our experimental glasshouse pots may not have provided sufficiently low resource levels to activate the mechanisms of competition required to detect a difference between provenances. However plants growing in the paired-plant pots grew on average 35% slower compared to singly grown plants, thus we believe our design provided a sufficient test of competition.

The lower growth rates in the presence of native-range rhizosphere microbial communities are consistent with the first prediction of the EICA hypothesis (i.e. escape from antagonists has led to loss of defences) and a parallel study that showed that New Zealand soils are more positive to *Trifolium* plant growth than native-range soils (McGinn 2015). The results are also consistent with a

previous study that found annual *Trifolium* in New Zealand have reduced mutualist association (Chapter 2). Plants from the non-native provenances appear to have altered performance post-naturalisation compared to native conspecifics, however these differences may not necessarily be adaptive and there is no evidence that they are related to competitive ability because neither performance nor competitive ability were greater in the non-native range. It is of course possible that other trade-offs are occurring in this system that were not within the reach of this study. For example, in New Zealand, *Trifolium* may be adapting increased hardseededness as protection against pathogens or grazers (Gravuer 2004). Alternatively, given the more extreme latitude, temperatures and UV in New Zealand, broader climate or UV tolerance (Hoffman *et al.* 2011) could be beneficial adaptations that contribute to these species successful spread and persistence in New Zealand (Boswell *et al.* 2003). Other general plant traits that can increase invisibility, particularly among annuals, include greater seed set and more rapid generation turnovers (Buswell et al. 2011; Kuester et al. 2014).

Alternatively, differences in growth may not be evidence of adaptive trade-offs at all, but rather a form of maternal effect in which growth is impaired because plants from the non-native range were naïve to the rhizosphere microbiota in the native-range soil treatments (Weiner *et al.* 1997; Bischoff & Müller-Schärer 2010). However, we reject the idea that maternal effects can fully explain the smaller size of non-native plants because a parallel pattern of inhibition would have been found among the plants from the native provenances when introduced to New Zealand rhizosphere communities—particularly given that these soils are certainly not enemy free (Skipp & Christensen 1983; Skipp & Watson 1987; Wratt & Smith 2013).

A final, potentially more parsimonious explanation is that the EICA hypothesis does not apply here and it cannot be considered a general explanation for the success of plant invaders. A recent review of the EICA literature by Felker-Quinn et al. (2013) revealed abundant evidence of adaptation among introduced plants, but found that support for EICA remains equivocal.

4.5.2 Growth rate versus competitive ability

The usefulness of growth rate as an outright indicator of plant invasibility, particularly due to the variation in growth among some taxa (Pan *et al.* 2011), has already been questioned. Additional studies have shown the importance of directly testing for post-naturalisation differences in performance in the context of limited resources (Leger & Rice 2003). A key strength of our study is that we directly measured the relative competitive ability of plants from non-native and native provenances using intra-specific competition experiments. While many tests of competition assume a positive correlation between growth rate and competitive ability (Blossey & Nötzold 1995; Handley *et al.* 2008; Franks *et al.* 2008), we found the opposite in this study: species having a larger difference

in inter-provenance growth rates when grown singly tended to have a smaller difference in relative competitive ability (i.e. when grown in pairs). This lack of correlation between measures of growth and measures of competitive ability suggests that caution should be taken when using measures such as growth rate or plant size as surrogates for competitive ability. Our meta-analysis of EICA competition studies (Table 4.2) shows further support for this lack of consensus between measures of growth and competitive ability. Only 13 EICA competition studies used both metrics of growth and competition; and of these, fewer than half show consistent results between increased growth and increased competitive ability (regardless of whether the results were positive, negative or neutral). We contend that it is not always possible to infer competitive differences from growth measures alone and that direct measures of competitive ability are needed to properly test for evidence of post-naturalisation change in invasive plant populations.

4.5.3 A better test of EICA

Our results indicate two ways to improve EICA studies that test for post-naturalisation adaptation in competitive ability. First, tests should use intra-specific pairings to compare the performance of plants from native and non-native provenances. In our meta-analysis, only three studies used an intra-specific test of competitive ability (Table 4.2). Performance measures in the presence of dominant heterospecifics in the new range are certainly valuable and informative, but such studies will be biased by intrinsic species-specific functional differences (Castro-Diez et al. 2014; Kiaer et al. 2013; Casper and Jackson 1997; Mangla et al. 2011) and thus cannot inform directly on post-naturalisation adaptations.

Second, we suggest that studies investigating invasive-plant competition should take a biogeographical approach and incorporate rhizosphere microbial communities from both the native and non-native ranges. Most EICA tests use soils that are sterilised, commercially sourced or neutral (i.e. not cultured by conspecifics), yet plant performance is intimately tied to interactions with belowground antagonists, mutualists, and saprophytes (Wardle *et al.* 2004; Inderjit & van der Putten 2010) and these synergistic components must be incorporated into plant-competition study designs. As the EICA hypothesis has mainly been developed from an aboveground point of view (Cipollini *et al.* 2005; Hull-Sanders *et al.* 2007; Doorduin & Vrieling 2011; Bekaert *et al.* 2012) it's now time for studies to integrate the role of rhizosphere microbial communities to better address the myriad potential effects of these communities on the post-naturalisation performance and competitive ability of non-native plants.

4.5.4 Conclusion

Although we found no evidence for increased competitive ability among a widely naturalised invasive genus, our results revealed an important discovery—that growth rate is not always an appropriate surrogate for competitive ability. We suggest that the use of (i) intra-specific-pairings, (ii) direct tests of competition, and (iii) the integration of rhizosphere microbial communities cultivated by conspecifics in each range will provide more powerful and informative EICA investigations of post-naturalisation differences in competitive ability.

Chapter 5

General Discussion

5.1 Thesis aims

The central goal of this thesis was to test whether post-naturalisation performance differences exist among *Trifolium* in New Zealand that would suggest successful invaders adapt to the conditions in the non-native range to become better competitors. To do this, I expanded the standard framework of one of the most widely tested but still equivocal explanations for invasive plant success—the evolution of increased competitive ability (EICA) hypothesis. Most tests of EICA have been conducted in a common-garden with commercial, sterilised or neutral soils (i.e. lacking the microbial communities cultivated by plants), yet rhizosphere communities are known to differ substantially between ranges and have a profound impact on plant performance, fitness and competition. In my investigations, I compared the performance of plants sourced from native and non-native provenances when grown in rhizosphere soils that had been cultivated *in situ* by conspecifics from each range. My whole-soil approach allowed me to further expand the original, enemy-focussed EICA framework by incorporating the effects of both antagonistic and mutualistic microbiota.

I sought to answer two fundamental questions: (i) Do non-native plants exhibit performance or trait differences that may be evidence for post-naturalisation adaptation? and (ii) Are there trends in post-naturalisation differences that correlate with invader success? I addressed these questions in three main experiments, each of which targets a previous gap in EICA experimental designs. First, I looked for inter-provenance differences in the degree of root mutualist association, as the benefits incurred by these associations suggest their loss will be just as likely to spur selection as the loss of antagonists. Second, I tested for inter-provenance differences in root flavonoid production, as root metabolites are pivotal to plant-microbe interactions in the rhizosphere—both as agents of defence and coordinators of rhizosphere mutualisms. Lastly, I sought to improve upon the standard EICA competition metrics by using a standardised index and intra-specific plant pairings to directly test for differences in competitive ability between plants from native and non-native provenances.

5.2 Key empirical results

5.2.1 Reduced association with rhizosphere mutualists

In Chapter 2, I asked whether plants from non-native provenances (New Zealand) have lower mutualist association with two of the most important rhizosphere endophytes—nitrogen-fixing rhizobia and arbuscular mycorrhizal fungi (AMF)—compared to plants from native provenances

(Spain and the UK). This prediction was based generally on contemporary recognition that microbes are dispersal limited (Hillebrand 2004; Pringle *et al.* 2009; Rout & Callaway 2012; Tedersoo *et al.* 2014) and was motivated specifically by soil surveys that show (i) that the agricultural rhizobia seeded into New Zealand pastures are generally not an ideal species-strain match for non-agricultural annuals of European origin (Howieson et al. 2006; Greenwood 1964; Yates *et al.* 2008), (ii) that the AMF common to New Zealand pastures (the likely point of entry for these *Trifolium* species (Gravuer *et al.* 2008)) are inefficient at sequestering nutrients (Powell 1979; Haynes & Francis 1990), and (iii) that *Trifolium* form "tripartite" associations wherein root endophytes rely on each other to enhance growth and fitness of all three partners.

My prediction for decreased mutualist association was supported most strongly in the case of rhizobia. Non-native provenances tended to have lower nodulation than native conspecifics in all soils and this difference was statistically significant in UK soils and in New Zealand soils. AMF colonisation was more variable than rhizobia, but on average colonisation was low for all plants in all soils (mean AMF colonisation was 19% across all plants). Only one inter-provenance comparison was significant—in New Zealand soil there was significantly lower colonisation among the New Zealand provenances compared to the UK provenances. All other AMF-association comparisons were not significant, including one in the opposite direction predicted (New Zealand plants had slightly higher AMF colonisation than Spanish plants when grown in Spanish soil).

Among non-native provenances, the overall lower nodulation and lower incremental growth benefit per rhizobia nodulation level suggests that *Trifolium* in New Zealand have developed decreased association with this mutualist. Decreased association could be beneficial in the context of naturalisation if (i) the rhizobia strains available in New Zealand are a poor match, or (ii) if the strains available colonise plants but are negative to their growth (i.e. parasitic). My glasshouse trials using rhizosphere soil cultivated *in situ* by conspecifics showed evidence for both scenarios in New Zealand soils. Nodulation was generally low in New Zealand soils for all plants, and *T. glomeratum* and *T. tomentosum* plants from both native and non-native provenances formed nodules characteristic of parasitism (non-functional nodules lack pigment) only in New Zealand soils. This supports previous work showing that although rhizobia from different effectiveness groups can form nodules on other host species, these rhizobia may not benefit the host plant and may actually out-compete any beneficial strains that may coexist in the soil (Pryor *et al.* 2004). In addition, the only plants that completely lacked rhizobia nodulation were *T. glomeratum* from the non-native provenance. Reduced association with rhizobia could also arise if (iii) plants from the non-native range have lost other capabilities that are necessary for the mutualism or (iv) the environment itself is less conducive to effective N-fixation. For example, Chapter 2 showed that although the colonisation by AMF was not significantly different between native and non-native plants in most soil treatments, the growth

benefit incurred by AMF was significantly lower among plants from the non-native provenances. Given that phosphorous is a requisite ingredient for successful N-fixation (Crush 1974), if plants are not deriving sufficient phosphorous from the soil directly or from AMF, this may hinder efficient N-fixation and contribute to the reduced association with rhizobia.

Surprisingly, although there was a significant difference in nodulation between native and non-native provenances in several treatments, and although regression models showed an overall positive growth benefit associated with increased nodulation, there was no evidence for physiological compensation for the lack of nodulation/benefit among plants from the non-native provenances and shoot-root ratios showed that plants from both provenances and in all soils invested more heavily in aboveground biomass—whereas we expect plants that lack rhizobia to invest more heavily in root biomass to access more soil nitrogen (Agren & Franklin 2003). There are a number of explanations for the lack of observed compensation. (i) Non-native *Trifolium* are able to acquire sufficient nitrogen even with limited rhizobia association. Although New Zealand soils are generally nitrogen-poor, congeners may help alleviate this effect, detailed below in Section 5.4.2. (ii) Compensation may be manifested in other traits, such as thinner, more branched root architecture (Seifert *et al.* 2009). Root architecture wasn't assessed in this study and if this form of compensation occurs in the *Trifolium* system, my growth-rate results could have been biased during root washing as the most delicate sections of root systems may have been lost. (iii) It is possible that compensation was occurring and shoot-root ratios were smaller for non-native provenances, but that the effect was confounded by the contribution of nodule weight among plants with high nodulation scores, as I did not weigh nodules separately (Agren & Franklin 2003). Although my ability to detect compensation may have been impaired by the aforementioned factors, the reduced association and mutualist benefit among these naturalised species suggests that neither mutualist limitations nor enhanced mutualisms are contributing to naturalisation success in New Zealand *Trifolium*.

5.2.2 Down-regulation of root flavonoids

In Chapter 3, I asked whether differences between rhizosphere microbial communities in the native-range and non-native-range have led to energetic trade-offs that have resulted in down-regulation of root flavonoids in favour of performance. Root flavonoids function to protect plants from biotic and abiotic stress and, in the context of plant-microbe rhizosphere interactions, flavonoids act much like hormones that respond to specific stimuli and direct microbial action. Thus, I predicted that, as a result of release from co-evolved rhizosphere antagonists and loss of highly compatible mutualist strains in the non-native range, plants from non-native provenances would produce lower concentrations and fewer types of flavonoids. I set up two treatments: sterilised soil (i.e. lacking biotic stimuli) to investigate constitutive (non-induced) flavonoid production, and unsterilised soil

cultivated by conspecifics in each range to investigate production of flavonoids specifically in response to rhizosphere microbiota in each range. In both treatments, I also assessed the richness and concentration of daidzein, an isoflavonoid associated with belowground defence and the rhizobia mutualism.

Flavonoid richness was generally lower among non-native provenances in both soil treatments and across all soil origins. Differences were most pronounced in the sterilised native-range soils, where flavonoid richness of non-native plants was about 2/3 the richness of native conspecifics. It's possible that any energetic benefit of producing fewer flavonoids is reallocated toward producing higher concentrations of each flavonoid (supported in this study by the similar total flavonoid concentrations). Alternatively, energetic reallocations may have benefitted a fitness characteristic, such as seed mass (Porter *et al.* 2011) or seed set, or enabled up-regulation in a metabolic pathway that was not investigated. In the *Trifolium* system, metabolites that function as protection against abiotic stress may be highly relevant, as temperatures on the islands can fluctuate greatly and the intensity of UV is much higher than it is in these species' native ranges (Hofmann *et al.* 2003; Allen & Lee 2006).

The richness of daidzein compounds was significantly positively correlated with rhizobia nodulation, providing some support for its role in the mutualism. However daidzein production differences between provenances varied in direction among soil origins and only one inter-provenance difference in daidzein production was significant—non-native provenances produced significantly fewer daidzein compounds compared to native conspecifics when grown in sterilised UK soil. The lack of trend in daidzein down-regulation among non-native provenances may relate to its function as a phytoanticipin, compounds that are produced independent of stimuli to help protect plants from attack from generalist enemies (Dakora & Phillips 1996), and/or its role as a defence against generalist soil fungal pathogens (Zilliken *et al.* 1984). Previous work has shown that even when plants experience enemy release from specialists, selection pressures sometimes lead to increases in generalist defences during naturalisation (Joshi & Vrieling 2005), presumably because constitutive production of a general defence compound helps to protect plants when they encounter occasional attack and/or novel enemies (Peñuelas *et al.* 2010), as even if non-native plants experience some level of enemy release; no soil is completely free of antagonists.

5.2.3 No evidence for increased competitive ability

In Chapter 4, I performed a direct test of competitive ability between native and non-native seed provenances when grown in soil cultivated *in situ* by conspecifics from either the native or non-native ranges. This whole-soil approach allowed me to consider in my analysis of competition the suite of interactions that occur in the rhizosphere of each range and may positively or negatively

affect plant competitive ability, including associations with plant growth-promoting endophytes such as arbuscular mycorrhizal fungi and nitrogen-fixing rhizobia, and antagonistic microbiota, including pathogenic fungi, bacteria, viruses, protists and parasitic nematodes. A key strength of this study is that it measured the relative competitive ability of non-native and native provenances using intra-specific competition experiments, whereas previous EICA tests use a heterospecific from each range as a "phytometer," which introduces the potential to confound species-specific interactive effects with post-naturalisation change. Overall, I found no consistent evidence for increased competitive ability as plants from non-native provenances grown in native-range soils had significantly slower growth rates. Surprisingly, despite their slower growth rates plants from the non-native provenances grown in competition with native conspecifics were no less competitive than these faster-growing natives. A positive correlation between performance (usually biomass production) in the absence of competition and competitive ability has been assumed under many previous tests of the EICA hypothesis (Felker-Quinn *et al.* 2013) and my finding (lower growth rates but not reduced competitive ability) demonstrates that this assumption is not generalizable.

5.3 Synthesis of major findings

This is the first test of EICA that explores the effects of both antagonists and mutualists on plant competitive ability, that integrates the role of rhizosphere microbiota from both ranges, and that uses congeners with a range of geographic distributions in the non-native range to test whether post-naturalisation differences can be predictive of invasive spread. Overall, I found support that plants from non-native provenances have diverged from native conspecifics in their association with a key rhizosphere mutualist, nitrogen-fixing rhizobia. I also found evidence that these plants are down-regulating production of root flavonoids, with evidence to specifically support lower richness in constitutively produced flavonoids, as differences were greatest in the sterilised soil treatments. However, I found no evidence that plants compensate for the loss of mutualist association. Nor did I find evidence that there is a growth benefit to flavonoid down-regulation. Non-native plants neither allocated more energy to root biomass nor showed overall greater growth rates. In addition, plants from non-native provenances did not show evidence of increased competitive ability. Thus, my first hypothesis was not supported. Neither did I find support for my second hypothesis, as the observed phenotypic differences between plants from native and non-native provenances were not correlated with the geographic success of each species on a regional or countrywide scale. In summary, although the New Zealand *Trifolium* differ from native conspecifics in several performance traits (decreased association with rhizobia, decreased growth-benefit from both rhizobia and AMF, and down-regulated flavonoids in native-range soils) that would suggest post-naturalisation adaptation in line with the first predictions of the EICA hypothesis, these differences were not correlated with the naturalisation success of these species. My findings are in accordance with a meta-analysis that was

published during the course of this work (Felker-Quinn *et al.* 2013), which found that there is abundant evidence for divergence in plant traits and performance (and specifically secondary chemistry) during naturalisation events, but that increased competitive ability among plant invaders is not a general trend.

5.4 Theoretical implications

This study serves as a theory-based test of a widely tested but equivocal explanation for why plants become invasive. A major strength of using the non-agricultural *Trifolium* of New Zealand as a model system to test the EICA hypothesis is that these species have a long naturalisation history that is not correlated with their geographic distributions (on a regional nor a countrywide scale). This allowed me to ask how divergence in certain traits post-naturalisation may or may not contribute to naturalisation success. In addition, this is the first study to incorporate the role of rhizosphere microbiota—pivotal players in plant performance, fitness and competition—from both the native and non-native ranges into multi-species tests of post-naturalisation performance and direct measures of competitive ability.

5.4.1 Misconceptions about size

Growth rate and biomass have been standard metrics with which to measure performance of non-native plants and indeed are the metrics of choice for testing many invasion-related hypotheses, including EICA. Yet, this study contributes to a growing body of evidence that size is not always a relevant factor in a plant's invasibility or likelihood of its naturalisation success in the non-native range (Bossdorf *et al.* 2005; Felker-Quinn *et al.* 2013). It has been suggested that the reason size has been historically misconstrued as an inherent characteristic of invaders is because the field data on invader size has consisted of non-random sampling (i.e. larger, problematic invaders are more likely to be studied) (Bossdorf *et al.* 2005). In this study, the two species that had the largest inter-provenance divergence in growth rate were *T. glomeratum* and *T. arvense*; despite their smaller size, these annuals are among the most widespread *Trifolium* in New Zealand (Figure 1.5; Figure 1.6).

5.4.2 The role of mutualists

The subset of plants that are widely naturalised have been purported to be those that do not depend heavily on mutualists (Richardson *et al.* 2000b; Mitchell *et al.* 2006), however, most plants associate with at least one rhizosphere endophyte—indeed, 80% of all plant taxa associate with at least arbuscular mycorrhizal fungi (Pringle *et al.* 2009). Although a handful of widely invasive plants are non-mycorrhizal—e.g. *Carpobrotus* spp. (Aizoaceae), *Myrica* spp. (Myricaceae) and *Lupinus* spp. (Fabaceae) (Brundrett 2008)—many of these taxa still host other root symbionts, including rhizobia or other plant-growth promoting endophytes. In addition, the characterisation of rhizosphere

mutualists is still in its nascence, so the extent of mutualisms and their benefit is unknown (Richardson *et al.* 2000a; Brundrett 2008; Baynes *et al.* 2012; Bever *et al.* 2015). Lost mutualisms are also likely to go undetected if the environmental conditions in the naturalised range fill the need provided by the lost mutualist. This may resolve the seeming dichotomy of successful invaders that host "tripartite" associations—these multi-partner relationships would seem to be more tenuous and the plants that host them more susceptible to invasion failure, however many legumes host these interdependent symbioses and members of this family are among the most widely naturalised genera in the world (ISSG 2015). This suggests that the role of one or more mutualists may be met in a different form in the non-native range or that plants are able to compensate for the loss. Although the invasive success of some plants clearly depends on the presence of a single mutualist partner (e.g. *Pinus radiata* and its ectomycorrhizal partner in New Zealand (Richardson *et al.* 1994), my findings suggest this scenario is probably not generalizable. In my study, *T. glomeratum* plants had low or no nodulation in New Zealand soils, yet this species is among the most widespread non-agricultural *Trifolium* in the country (Figure 1.5) and was the most common of the seven species in the New Zealand study area (Figure 1.6).

5.4.3 Shifting biochemical profiles

EICA investigations of plant biochemistry have thus far been dominated by quantifications of foliar compounds or indirect measures of palatability or herbivore fitness. A recent meta-review (Felker-Quinn *et al.* 2013) revealed that although many plants alter their biochemical profiles post-naturalisation, there are no significant trends suggesting foliar defences decrease overall in the non-native range. However no analogous meta-analysis has been done with belowground chemistry because these investigations are lacking. The generalisability of root flavonoid down-regulation as a beneficial post-naturalisation shift is questionable however as some studies suggest that rhizosphere antagonists "catch up" with plant hosts or accumulate over time (Diez *et al.* 2010). While reallocations of flavonoid production may provide a short-term fitness advantage, the inability to produce specific flavonoids (i.e. if low flavonoid richness becomes "fixed" in a non-native population) could potentially hinder plant performance or limit further spread if plants are poorly defended. Microbes are dispersal limited (Tedersoo *et al.* 2014), but many still colonise outside their ranges and a recent study suggests that many of those naturalisations go undetected (Litchman 2010). If antagonists are introduced from the native range, or in fact from any other location, negative soil biota may accumulate over time, cancelling out any early benefit of enemy release or fitness trade-offs in line with EICA. Similarly, down-regulating production of mutualist-related metabolites may decrease a plant's ability to form relationships with novel biota, or with a lost co-evolved mutualist if it becomes available later (Seifert *et al.* 2009). In addition, because the metabolites produced by plants to stimulate different mutualists can be chemically similar, down-regulation of one part of the

flavonoid pathway can have concurrent implications for other mutualists (Paszkowski 2006). Alternatively, the multi-functional nature of some flavonoids (Weston & Mathesius 2013) may lead to flavonoid preservation even when non-native plants experience enemy release or lost mutualists—however this was not supported in my study.

5.4.4 Do congeners contribute to invader success?

New Zealand soils are generally nutrient-poor, which has been one suggested explanation for why legumes that host nitrogen-fixing symbionts have been so successful at out-competing resident flora (Richardson *et al.* 2000a; Allen & Lee 2006). But while hosting a nutrient-acquiring mutualist may provide an innate advantage to colonisers if the new environment is nutrient-poor, the lack of mutualist availability can prevent establishment or limit dispersal. In this study, I found support for reduced mutualist association among non-agricultural *Trifolium,* yet these species are successful in New Zealand, albeit to varying degrees by species (Appendix A.3). The loss of mutualist association without evidence of trade-offs or compensation was surprising because these species are successfully naturalising in a country where soils are generally nitrogen- and phosphorous-poor (Sarathchandra *et al.* 1984; Boswell *et al.* 2003). A potential explanation is that these species may receive the benefits (specifically nitrogen) provided by the rhizobia mutualist indirectly from congeners. Species of agricultural *Trifolium* are widespread throughout New Zealand and strains of agricultural rhizobia are seeded regularly into New Zealand pastures (Lowther & Kerr 2011), and have now naturalised far beyond pastures. Thus, wherever there are agricultural *Trifolium*, nitrogen is unlikely to be a limiting nutrient. This speculation is supported by my field observations during soil and seed sampling, as the non-agricultural species were always closely associated with agricultural *Trifolium* in the non-native range. This speculation is also supported by the results of the competition analysis (Chapter 4) wherein the competitive ability of non-native provenances was similar to that of native conspecifics despite them being significantly smaller than native conspecifics in all treatments. In the paired-plant competition pots, the native plant may have been contributing to the growth of the non-native plant by supplying excess nitrogen to the pot. This has an important implication for naturalisation patterns—where invading congeners naturalise together, they may be able to provide services (e.g. shared mutualists, rhizosphere cultivation, provision of limiting nutrients) that enhance the spread of species that might otherwise be limited. This is somewhat akin to the "invasion meltdown" hypothesis (Simberloff & Holle 1999), and may help to explain why plants that should theoretically be limited by mutualists can naturalise successfully in their absence.

5.4.5 Competition in the non-native range

The EICA hypothesis predicts that fitness trade-offs result in plants with greater competitive ability and that this feature is what enables them to become invasive (Blossey & Nötzold 1995). However,

adaptation for increased competitive ability should not be expected in every plant invasion scenario and is perhaps the exception rather than the rule. Invaded environments tend to be those where resources are rich, competitors are scarce or the intrinsic level of competition is lower than in the invader's native range (Alpert et al. 2000; Whitney and Gabler 2008), so there may actually be greater selective pressure against competitive ability in many invasion scenarios. Decreased competition in the non-native range has been suggested to explain the naturalisation success of *Centaurea stoebe* (Asteraceae), which escapes from competitive plant neighbours in the non-native range (Sun *et al.* 2014). In addition, it has been suggested that in stressful or low-resource environments, species may evolve to separate resource capture from growth; as in the case of invasive *Hieracium* spp. (Asteraceae) in New Zealand hill country, which grow on poor soils with pasture species (including *T. repens)* but do not experience competitive effects (Scott & Sutherland 1993). Therefore competitive ability, like selection for any plant trait, is likely to be context-dependent and driven by the conditions (enemies, mutualists, resources, competition, etc.) to which the non-native plant is subjected.

5.4.6 Rhizosphere microbiota dynamics

Rhizosphere microbiota are also subject to fundamental evolutionary processes and thus the interactive effects and selective pressure of these communities on non-native plants is likely to change over time (Rout & Callaway 2012; Coats & Rumpho 2014). To understand how these communities impact invasions therefore requires parallel investigations of differences in plant performance and fitness over time, as well as differences in the rhizosphere communities with which they interact. This can include host-switching of pathogens, supported by studies that show enemies accumulate on invaders over time after they naturalise (Diez *et al.* 2010), or changes in symbiont-host interactions (including preference) over time (Seifert *et al.* 2009; Porter *et al.* 2011). Because of the rapid generation times and high rates of mutation of most microbes (Coats & Rumpho 2014), genetic-based changes among these rhizosphere microbial communities are likely to progress at even faster rates than for their plant hosts. In addition, evolutionary change among microbial communities may present more varied outcomes because bacteria can exchange genetic information via horizontal gene transfer and mycorrhizal systems create underground networks that link entire plant communities wherein genetic information is shared (Denison & Kiers 2004b; Weir 2006; Rout & Callaway 2012).

There is already evidence for genetic change in rhizosphere microbial communities in New Zealand. Non-native *Rhizobium leguminosarum* that has been introduced for agricultural legumes has evolved to nodulate with native New Zealand legumes, presumably as a result of horizontal gene transfer with native rhizobia species (Weir 2006). Analogous transfers of genetic information may be

occurring for the biovar investigated in this thesis, *R. leguminosarum* bv*. trifolii,* and could help to explain why annuals from different rhizobia effectiveness groups nodulated in most soil types from New Zealand, despite the likelihood that the field inoculants were dominated by agricultural strains best suited to New Zealand's widespread perennial *Trifolium* spp. The continual seeding of agricultural rhizobia into New Zealand pasturelands has likely increased the genetic richness of these rhizobia communities, which is supported by a parallel study that showed total rhizobia richness was similar between New Zealand and the UK (McGinn 2015). Gene exchange among these rhizobia communities in New Zealand may have enabled agricultural strains to expand their compatibility so that they can nodulate with non-target *Trifolium* species—even if the strains are not highly beneficial to plant growth or even parasitic, as I found in this study. This would have important implications for invasion biology because continual introduction of rhizosphere mutualists for agricultural plants can potentially expand the genetic richness of naturalised strains as well as native rhizosphere mutualists.

Investigating how microbial interactions may differ between native and introduced ranges could also reveal new patterns of adaptation among plants and the microbes they host. Rhizosphere mutualists such as AMF can alter plant morphology, allometry, phenology, the production of secondary metabolites, and fitness—including re-allocation of reproductive strategies (Johnson et al., 1997), suggesting great potential for plants to respond and potentially adapt to differences when they encounter different microbial communities outside their native ranges. It has been suggested that invasive plants frequently face a higher incidence of parasitism during naturalisation (Thrall et al., 2007) but that the phenomenon goes unnoticed simply because it is masked by concurrently higher resource levels (Karst et al., 2008), enemy release (Joshi and Vrieling, 2005) or reduced competition (van der Putten and Peters, 1997). Alternatively, escape from parasitic co-evolved mutualists in the native range could constitute an unexplored form of enemy release for invasive plants, with concurrent potential adaptations in line with EICA predictions of resource re-allocation. Indeed, higher rates of parasitism among rhizosphere biota could help to explain why plant-soil feedbacks are generally more negative in the native range (Beckstead and Parker, 2003, van Grunsven *et al.*, 2009, Reinhart & Callaway, 2004).

Before being able to compare how native and invaded plant populations interact differently with their microbial communities, it would be prudent to expand our understanding of how plants interact with rhizosphere microbial communities throughout their native ranges, effectively quantifying the range phenotypic responses plants may have when grown with different microbial communities. We can predict that microbial communities will be more similar in a proximal geographic area and thus we would expect that this would attenuate the effects of "enemy release" or "lost mutualisms." Although it was not feasible in this study to perform fully factorial comparisons in all soils (i.e.

Spanish plants grown in UK soils and UK plants grown in Spanish soils), we can speculate that plants grown in an alternate native-range soil could either perform better (i.e. grow faster or larger) because the soil antagonists are naïve or they may perform worse (i.e. grow slower and/or be less colonised) because the mutualists available are not optimal strain-genotype pairings. Biogeographical experiments such as these can broaden our understanding of the potential responses of plants when exposed to different microbial communities, including those outside their native ranges.

5.4.7 Benefits of utilising a whole-soil approach

It has been suggested that tests of the EICA prediction for reduced defences and increased fitness have been limited by experimental designs that only include one or a few antagonist species, whereas the mechanisms that affect resistance may differ greatly depending on whether the antagonist is a specialist or generalist (Bossdorf *et al.* 2005; Hull-Sanders *et al.* 2007) and can be intimately related to the availability of rhizosphere mutualists (Pieterse *et al.* 2014). This highlights an important advantage to using a whole-soil approach wherein the effects of antagonists, mutualists and neutral biota are evaluated in parallel. Once the nature of any post-naturalisation performance differences have been rigorously tested and the variation among different populations established (e.g. variation inherent in environmental clines (Colautti *et al.* 2009)), parsing out the effects of individual microbial players (e.g. by serial dilutions that break down rhizosphere microbial communities into progressively smaller fractions) and secondary chemistry (e.g. root compounds and allelopathic exudates) can be used to elucidate the specific mechanisms responsible.

5.5 Study limitations and recommendations

The value of hypotheses such as EICA is that they attempt to identify generalizable trends among species that can help identify the factors that contribute to invader success, thus helping to better predict invasion trajectories or future naturalisation events. Given the unexpectedly mixed findings in the New Zealand *Trifolium* system, the following suggestions may further improve the EICA framework.

1. Incorporate greater within-provenance replication. There can be great variation in performance and traits among plant taxa and even among plant populations of a single species and this can obscure our ability to differentiate among population-level variation, phenotypic plasticity and post-naturalisation change (Pan *et al.* 2011). In this thesis I specifically use species as the level of replication to look for evidence of divergence between plants from different provenance origins, however the seed populations chosen in each provenance may not represent well the genotypes of the respective provenances. Although the intra-provenance variability is predicted to "even out" as a result of using multiple species, a limitation of my study design is that all plants and soils from the

New Zealand provenance were selected from one study region and thus the seed and soils may not broadly represent the genotypes and conditions of the non-native range.

Greater replication at the within-provenance level would also have provided the statistical power to evaluate the variation in plant response to each soil rhizosphere community in each provenance. Capturing variability in plant genotype response to a common rhizosphere community was not an objective in my study, where soil sites formed the glasshouse pot replicates and my focus was on discerning divergence in seed provenance, however I believe there is great value in future tests incorporating this level of replication in order to better understand how plant-microbe interactions differ by genotype. Quantifying the variation in plant response to different microbial communities would also provide better estimates of the effect sizes necessary to detect post-naturalisation change in plant populations—and help future studies avoid both Type I and Type II errors.

2. Measure multiple plant traits in parallel against fitness traits. My results support a recent meta-analysis of EICA literature, which found abundant evidence for post-naturalisation trait shifts among invasive plants, but not concurrent benefits to fitness or competitive ability (Felker-Quinn *et al.* 2013). This appears to be in discordance with the parameters of Optimal Defence Theory (Herms & Mattson 1992; Ridenour *et al.* 2008), however the reason for the apparent lack of trade-offs may be that the traits involved in energetic reallocations simply were not the ones studied (Bossdorf *et al.* 2005; Colautti *et al.* 2009). Multiple traits are likely to be shifting in parallel as a result of a variety of evolutionary forces (Abhilasha & Joshi 2009; Abela-Hofbauerová & Münzbergová 2011), which include natural selection but also stochastic mechanisms, such as genetic drift (Reznick & Ghalambor 2001). Investigating a wider range of plant traits, and targeting traits specific to the study system, may help to elucidate whether post-naturalisation trait shifts with fitness value are indeed a generalizable phenomenon among invaders. A limitation of this study in hindsight is that I chose to use plant growth as a primary metric of performance; plants were harvested as soon as they began to form buds (indicating a shift from growth to reproduction) and thus I was not able to also analyse reproductive traits such as flowering phenology, seed output or seed size. Other post-naturalisation traits that might be more relevant than growth in the *Trifolium* system could be hardseededness, as grazing pressure is high and clover pathogens are abundant in New Zealand (Skipp & Christensen 1983; Skipp & Watson 1987; Wratt & Smith 2013), or tolerance to UV or drought (Hofmann *et al.* 2011).

In this thesis, I did not find significant correlations between geographic distribution and any of the factors of interest (mutualist association and benefit, flavonoid production, competitive ability). This suggests that the differences in these species' distributions in New Zealand can be explained by other factors, such as differences in their rates of introduction as seed contaminants (Gravuer *et al.*, 2008),

habitat preferences, or differing degrees of escape from antagonistic biota in the soil—particularly because the strength of enemy release has been shown to be stronger than the benefit of mutualists in the naturalisation of another legume, *Robinia pseudoacacia* (Callaway *et al.*, 2011).

Finally, I suggest that there is great benefit in investigating how plant traits differ *in situ* (i.e. when plants are interacting with communities in each range), because chemical differences may not always be the most informative metric. For example, in a study specifically aimed at evaluating the generalisability of EICA, secondary chemical production was no different between plants from the native and non-native ranges, however the fitness of one of the insects studied was significantly higher when it fed on non-native plants (Hull-Sanders *et al.* 2007). This highlights the inherent limitations in our understanding of the role of different chemical compounds and the importance of directly investigating community interactions.

3. Longer-term studies. Studies often take place in a single year or season and do not incorporate the role of phenological differences in environmental conditions and other biota, including how populations of antagonists and mutualists may fluctuate over time. Agrawal *et al.* (2005) suggested there may be "invasion opportunity windows," periods of time when non-native plants experience periodic enemy release and extend their distributions. Most studies, including this one only look at a single season of data, even though it is well known that factors such as reproductive output and pollinator abundance can fluctuate by several orders of magnitude between seasons or years. Analogous patterns surely exist in the rhizosphere as well, but have not been systematically tracked.

4. Incorporate genetics and ecology. Soil surveys that incorporate microbial genotyping are enabling coarse differentiation of the soil microbiota between locations (Birnbaum *et al.* 2014; Tedersoo *et al.* 2014), however a phylogenetic approach does not necessarily inform on how plants and microbes will interact *in situ*. Recent global studies of microbial diversity and high-resolution genetic studies show that these analyses are limited in their ability to detect subtle differences in functional traits among microbes. For example, an analysis of two rhizobia strains—one an effective nodulator and the other a knock-out—revealed that their 16S rRNA sequences were identical, despite differences in their symbiotic and chromosomal replicons (Yates *et al.* 2008). These two strains would not be differentiated in a standard genetic analysis, yet are functionally disparate. This highlights an important lesson from an ecological perspective; even where soil surveys have been conducted, metrics of genetic similarity in the microbial communities in two areas communicate only a partial story—ecological studies are still needed to examine the functionality of these genotypes and how they interact with different plant genotypes. Given the inherent dynamism and taxa-specific outcomes of plant-microbe interactions, there is great value in performing both genetics-based and ecology-based testing.

Lastly, a future direction for management-focussed invasion studies may be the experimental manipulation of rhizosphere communities to control plant invasions. Plant performance and competitive ability are intimately tied to the microbial communities in the rhizosphere (Coats & Rumpho 2014), and agricultural systems have long capitalised on the increased fitness of plants after seeding soils with compatible AMF or rhizobia. Introducing a single obligate rhizosphere mutualist (ectomycorrhizae) enabled the widespread naturalisation of *Pinus radiata* (Pinaceae) in New Zealand within only a few decades (Richardson *et al.* 1994)—illustrating how even a single taxon can completely alter invasion outcomes. Efforts are already underway to characterise the taxonomic and functional diversity of microbiota in different biomes on a global scale using metagenomic approaches (http://www.earthmicrobiome.org). Better understanding of rhizosphere communities and how they might be manipulated to control the spread of non-native plants—such as by increasing the competitive ability of natives or by making habitats more robust to invasion—could provide a new tool in the management of noxious weeds.

5.6 Conclusions

My findings indicate that plants from the non-native provenance (New Zealand) of a widely naturalised genus (*Trifolium)* have diverged from native provenances in three ways: (i) decreased growth rates compared to plants from non-native provenances; (ii) decreased association and benefit from root mutualists and (iii) down-regulated production of root flavonoids. However, I found no evidence for physiological compensations in the form of greater allocation to root biomass nor increased competitive ability that would suggest these differences may be associated with fitness trade-offs. Most importantly, there was no evidence that trait divergence was correlated with the naturalisation success of these species. Thus, the EICA hypothesis was not fully supported in this system. This work contributes to a mounting body of work supporting substantial post-naturalisation trait shifts without discernible benefit to plant fitness. Thus, while trait divergence may be common among invaders, it is not necessarily a generalizable mechanism to explain invasion outcomes. This study also substantiates two important lessons relevant to all future EICA studies: (1) plant growth rates and size are not always appropriate metrics for gauging plant invasibility; and (2) the lack of correlation between measures of growth and competitive ability suggests that growth and biomass are not appropriate surrogates with which to quantify competitive ability.

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Appendix A

Sampling locations and geographic distributions by species

A.1 Field site location details for the rhizosphere soil used to inoculate the glasshouse pots

Species	Country	Location	Latitude	Longitude	Local abundance*	Mean pH
T. arvense	Spain	Parador de Oriel, Aragon	42.52767	-00.53161	1	6.12
	Spain	Benabarre, Huerrios Mtns, Aragon	42.13871	00.47665	2	6.49
	Spain	Benabarre, Huerrios Mtns, Aragon	42.16830	00.45163	2	5.61
	Spain	Blanes, Catalonia	41.66857	02.76699	1	7.77
	Spain	El Port de la Selva, Catalonia	42.32964	03.19938	2	7.68
	UK	Bournemouth, Monkey Island	50.71949	-01.85750	1	5.36
	UK	Gosport, Browndown	50.79236	-01.19320	2	7.14
	UK	Devon, near Torquay	50.45835	-03.49138	0	4.81
	UK	Gower, Pennard Burrows	51.57602	-04.09137	0	5.24
	UK	Kenfig	51.51550	-03.72777	1	6.47
	NZ	Christchurch, Canterbury	-43.53654	172.60981	1	5.54
	NZ	Kaitorete Spit, Banks Pen.	-43.82550	172.69896	1	5.31
	NZ	Birdlings Flat, Banks Pen.	-43.81554	172.69999	2	5.26
	NZ	New Brighton, Canterbury	-43.52554	172.72266	1	6.00
	NZ	South Brighton, Canterbury	-43.51955	172.71967	0	7.03
T. campestre	Spain	Zubillaga, Alaba, Basque Country	42.71492	-02.9784	2	7.70
	Spain	Pancorbo, Castilla y León	42.63908	-03.10591	2	7.52
	Spain	Barcina del Barco, Castilla Y León	42.78152	-03.22899	2	7.62
	Spain	Rio Ebro, Castilla y León	42.76448	-03.18876	2	7.95

	Spain	Embalse de Sobrón, Basque Country	42.76807	-03.10081	2	7.60
	UK	Gosport, Browndown	50.79231	-01.19333	2	7.34
	UK	Gosport, Browndown	50.79346	-01.19466	2	7.25
	UK	Swansea, Crymlyn	51.62422	-03.84303	0	6.75
	UK	Devon, near Chudleigh	50.61418	-03.62361	0	6.58
	UK	Kenfig, near Sharkham Point	51.50608	-03.74308	2	7.26
	NZ	Christchurch, Canterbury	-43.53654	172.60981	0	6.68
	NZ	Chorlton Road, Banks Pen.	-43.67533	173.04443	0	5.72
	NZ	Western Valley Road, Banks Pen.	-43.74696	172.79556	1	5.71
	NZ	Streeters Road	-43.73616	172.62569	1	4.73
	NZ	Big Hill Road, Banks Pen.	-43.70170	173.06485	2	5.79
T. glomeratum	Spain	Merindad de Valdivielso, Castilla y León	42.82221	-03.53400	1	6.54
	Spain	Panizares, Castilla y León	42.80066	-03.47124	0	7.07
	Spain	Curbo de Burebas, Castilla Y León	42.64080	-03.20654	1	7.70
	Spain	Navas de Bureba, Castilla Y León	42.68304	-03.32516	0	6.90
	Spain	Pino de Bureba, Castilla Y León	42.70662	-03.42762	0	7.00
	UK	Bournemouth, Monkey Island	50.71935	-01.85740	1	5.35
	UK	Gosport	50.79224	-01.17752	1	5.48
	UK	Devon, near Torquay	50.46021	-03.48866	1	5.29
	UK	Devon	50.54711	-03.57068	2	5.59
	UK	Devon, south of Berryhead	50.38207	-03.49998	0	6.84
	NZ	Okains Bay, Banks Pen.	-43.70449	173.04723	1	6.18
	NZ	Kaitorete Spit, Banks Peninsula	-43.82550	172.69896	0	5.87
	NZ	Western Valley Road, Banks Pen.	-43.74696	172.79556	1	6.19
	NZ	New Brighton, Canterbury	-43.52554	172.72266	0	6.04
	NZ	Heathcote Quarry, Banks Pen.	-43.57186	172.71675	1	6.51
T. micranthum	UK	Bournemouth	50.72027	-01.84962	1	6.87
	UK	Gosport marsh	50.77955	-01.14607	2	5.59

	UK	Gower, Broadpool	51.59798	-04.15196	2	4.82
	UK	Gower, Port Eynon	51.54380	-04.21111	2	6.94
	UK	Gower, Loughour Estuary	51.63116	-04.13500	1	6.73
	NZ	Christchurch, Canterbury	-43.49827	172.63873	1	5.33
	NZ	South Brighton, Canterbury	-43.53237	172.73447	1	5.41
	NZ	Lincoln, Canterbury	-43.64350	172.46848	2	5.02
	NZ	Christchurch, Canterbury	-43.52733	172.57504	2	5.78
	NZ	Lincoln, Canterbury	-43.64065	172.48551	2	5.25
T. ornithopodioides	UK	Bournemouth	50.72029	-01.84949	1	6.21
	UK	Gosport	50.79211	-01.18059	0	7.08
	UK	Gower, Port Eynon	51.54385	-04.21126	2	6.99
	UK	Gower, Loughour Estuary	51.63081	-04.13473	2	6.82
	UK	Kenfig, near Sharkham point	51.50681	-03.74062	2	7.48
	NZ	Little River, Banks Pen.	-43.76945	172.79064	1	4.49
	NZ	Lake Forsyth, Canterbury	-43.80974	172.72339	2	4.85
	NZ	Governors Bay, Banks Pen.	-43.65182	172.65638	2	4.56
	NZ	Squally Bay, Banks Pen.	-43.89392	172.91484	1	5.14
	NZ	Lincoln, Canterbury	-43.64350	172.46848	2	4.70
T. striatum	Spain	Parador de Oriel, Aragon	42.52767	-00.53161	0	6.08
	Spain	Benabarre, Huerrios Mtns, Aragon	42.13871	00.47665	1	5.96
	Spain	Sant Pere de Rodes, Catalonia	42.32933	03.15826	0	5.64
	Spain	Girona, Catalonia	42.29192	03.15193	1	6.85
	Spain	Girona, Catalonia	42.36454	03.02926	2	5.82
	UK	Bournemouth, Monkey Island	50.71936	-01.85817	1	5.14
	UK	Gosport	50.79219	-01.18068	0	6.96
	UK	Devon, south of Berryhead	50.38211	-03.49983	0	6.85
	UK	Gower, Broadpool	51.59786	-04.15189	2	5.17

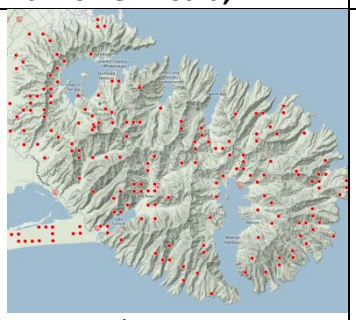
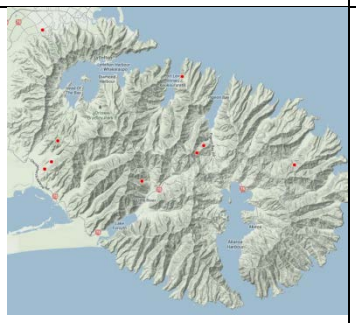
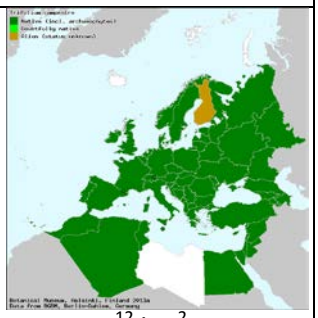
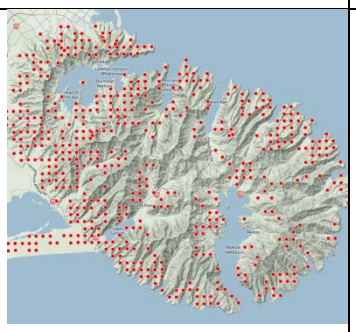
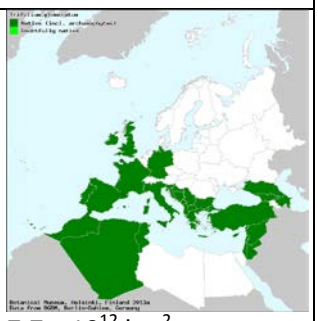
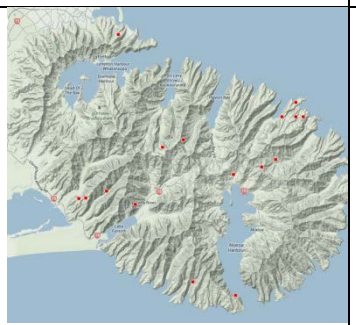
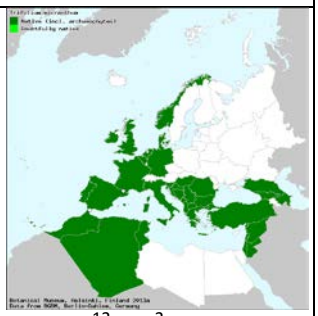
	UK	Gower, Pennard Burrows	51.57591	-04.09119	0	5.05
	NZ	Birdlings Flat, Banks Pen.	-43.81554	172.69999	1	5.24
	NZ	Heathcote Quarry Track, Banks Pen.	-43.57186	172.71675	1	5.41
	NZ	Governors Bay, Banks Peninsula	-43.63209	172.65205	2	5.27
	NZ	Ataahua Domain, Canterbury	-43.77608	172.64566	0	4.44
	NZ	Big Hill Road, Banks Pen.	-43.70170	173.06485	2	5.89
T. tomentosum	Spain	Pancorbo, Castilla Y León	42.63908	-03.10591	0	7.87
	Spain	Larrazubi, Alaba, Basque Country	42.74847	-03.05093	1	8.04
	Spain	Miraveche, Castilla y León	42.67663	-03.19665	1	7.51
	Spain	Busto de Bureba, Castilla y León	42.65952	-03.26588	2	7.34
	Spain	Navas de Bureba, Castilla y León	42.68304	-03.32516	0	7.43
	NZ	Kaitorete Spit, Banks Peninsula	-43.82550	172.69896	1	5.26
	NZ	Ataahua Domain, Canterbury	-43.77608	172.64566	0	5.50
	NZ	New Brighton, Canterbury	-43.51184	172.71680	2	5.73
	NZ	South Brighton, Canterbury	-43.51955	172.71967	1	5.92
	NZ	South Brighton, Canterbury	-43.51676	172.73383	1	5.26

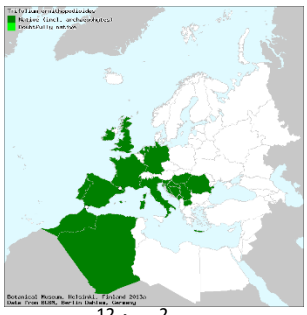
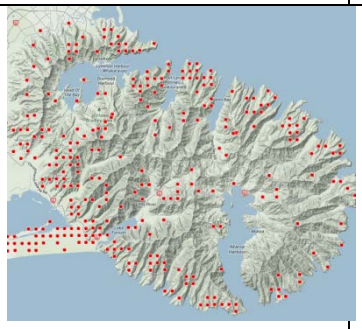
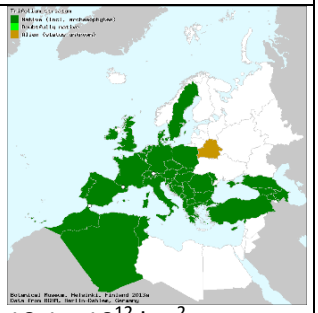
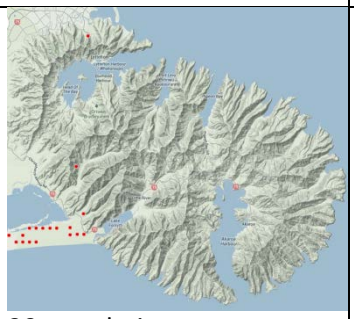
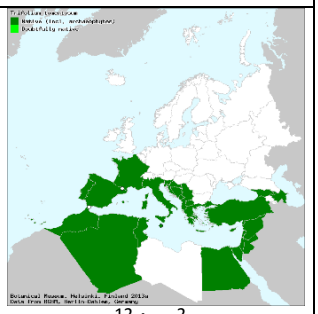
*Local abundance was estimated using a modified DAFOR (Dominant, Abundant, Frequent, Occasional, Rare) scale where 0 = occasional, 1 = frequent, 2 = abundant.

A.2 Seed source locations for the seven species of *Trifolium*

Species	Country	Region	Source	Latitude	Longitude	Collected	Chp used
T. arvense	UK	NA	Herbiseed	NA	NA	NA	2,3,4
	Spain	Blanes, Catalonia	Field	41.66857	02.76699	June 2012	2,3,4
	New Zealand	Kaitorete Spit, Canterbury	Field	-43.82550	172.69896	Feb .2012	2,3,4
T. campestre	UK	Crymlyn Burrows, Wales	Field	51.62422	-03.84303	June 2012	2,4
	Spain	Gorliz Beach, Basque Country	Field	43.41476	-02.94018	June 2012	2,4
	New Zealand	Banks Peninsula, Canterbury	Field	-43.69072	172.64035	Feb. 2012	2
	New Zealand	Banks Peninsula, Canterbury	Field	-43.70170	173.06485	Feb. 2012	4
T. glomeratum	UK	Bournemouth, England	Field	50.71935	-01.85740	June 2012	2,3
	Spain	El Port de la Selva, Catalonia	Field	42.32964	03.19938	June 2012	2,3
	New Zealand	Kaitorete Spit, Canterbury	Field	-43.82550	172.69896	Feb. 2012	2,3
T. micranthum	UK	Port Eynon, Gower, Wales	Field	51.543807	-04.21111	June 2012	2
	New Zealand	Lincoln, Canterbury	Field	-43.64350	172.46848	Feb. 2012	2
T. ornithopodioides	UK	Port Eynon, Gower, Wales	Field	51.543807	-04.21111	June 2012	2,3
	New Zealand	Banks Peninsula, Canterbury	Field	-43.89392	172.91484	Dec. 2011	2,3
T. striatum	UK	Bournemouth, England	Field	50.719356	-01.85740	June 2012	2,3,4
	Spain	Huerrios Mtns, Aragon	Field	42.14666	-00.47159	June 2012	2,3,4
	New Zealand	Banks Peninsula, Canterbury	Field	-43.63209	172.65205	Feb. 2012	2,3,4
T. tomentosum	Spain	Castilla Y León	Field	42.561784	-05.77344	June 2012	2,3
	New Zealand	Banks Peninsula, Canterbury	Field	-43.77608	172.64566	Feb. 2012	2,3

A.3 Geographic distributions for the seven species of *Trifolium*

	New Zealand*	Banks Peninsula, NZ†	Native-range area¥
T. arvense	 83 (10 x 10 km) NZMS260 grids	 199 populations	 26.6 x 10^{12} km^2
T. campestre	 46 (10 x 10 km) NZMS260 grids	 9 populations	 20.1 x 10^{12} km^2
T. glomeratum	 44 (10 x 10 km) NZMS260 grids	 564 populations	 7.7 x 10^{12} km^2
T. micranthum	 23 (10 x 10 km) NZMS260 grids	 16 populations	 10 x 10^{12} km^2

T. ornithopodioides	 26 (10 x 10 km) NZMS260 grids	 5 populations	 5.4 x 10^{12} km^2
T. striatum	 44 (10 x 10 km) NZMS260 grids	 301 populations	 10.1 x 10^{12} km^2
T. tomentosum	 21 (10 x 10 km) NZMS260 grids	 30 populations	 11.4 x 10^{12} km^2

* Data from Gravuer 2004; images from Landcare Vegetation Survey Database, nvs.landcareresearch.co.nz

† Data from Wiser et al. 2001; images from Landcare Vegetation Survey Database, nvs.landcareresearch.co.nz

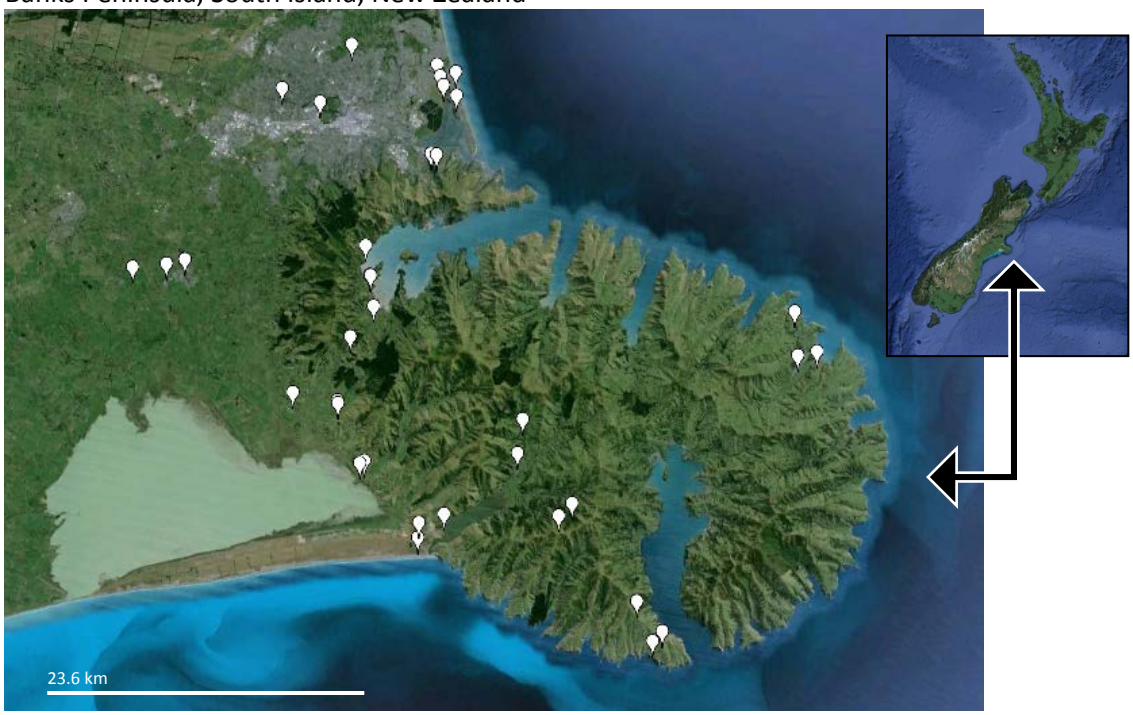
¥ Data from Gravuer 2004; images from Euro-Med Plant Base, ww2.bgbm.org/EuroPlusMed (green represents native range; brown represents non-native area)

A.4 Soil sampling sites (Chp 2-4)

Northern Spain

Southern UK

Banks Peninsula, South Island, New Zealand



Soil sampling sites for the native range (A) northern Spain and (B) southern UK and the non-native range (C) Banks Peninsula, South Island, New Zealand for the seven species of *Trifolium* used in this study. Maps were created using Google Earth ver. 7.1.2.

Appendix B

Glasshouse experiments

B.1 Germination conditions

Germination conditions used in the light- and temperature-controlled cabinets for the seven species of *Trifolium* in this thesis.

Species	Days in paper at 4° C	Light: Dark	Temperature range	Weeks to transplant
T. arvense	2	8:16	16-18° C	4
T. campestre	4	8:16	16-18° C	4
T. glomeratum	2	8:16	16-18° C	3
T. micranthum	2	12:12	10-12° C	3
T. ornithopodioides	2	12:12	10-12° C	4
T. striatum	0	8:16	16-18° C	3
T. tomentosum	0	8:16	16-18° C	4

B.2 Glasshouse soil properties

Sandy background soils formed 90% of the volume of each glasshouse pot. In New Zealand, soil fractions were sourced commercially and mixed to achieve a sandy soil with good drainage. In Europe, soils were collected from a field location in The Netherlands. Soils were sterilised by autoclaving (two rounds of 20 min at 121° C) in New Zealand and by gamma irradiation (>25 KGray) in Europe. Organic matter and nitrogen levels were comparable and no evidence of manganese toxicity was discernible in the autoclaved soils. * me = a measure of cation-exchange capacity (milliequivalent of hydrogen per 100 g of dry soil).

	New Zealand		Europe
Soil property	**Pre-autoclave**	**Post-autoclave**	
pH	5.9	6.10	6.23
Total nitrogen (%)	N/A	0.10	0.06
Total carbon (%)	N/A	1.20	1.00
Carbon:nitrogen ratio	N/A	11.50	16.60
Total % organic matter	N/A	2.00	2.97
Olsen P (mg/L)	10	8	
K (me*/100g)	0.28	0.45	
Ca (me*/100g)	2.6	3.4	
Mg (me*/100g)	0.56	0.66	
Na (me*/100g)	0.12	0.23	
CEC (me*/100g)	5	6	
Total base saturation (%)	71	76	
Mn (mg/kg)	152	189	

Appendix C

Quantification of rhizosphere mutualists (Chp 2-4)

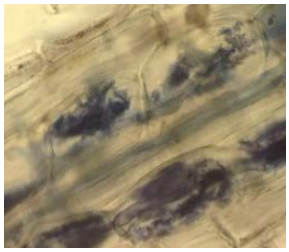
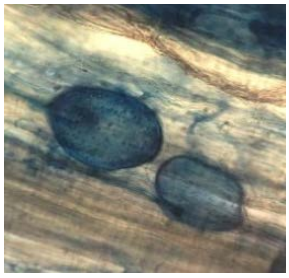
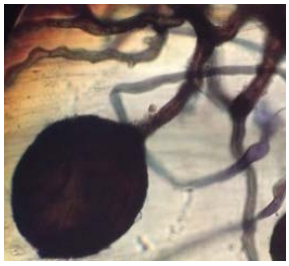
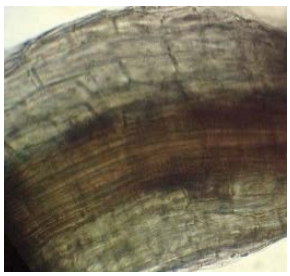
C.1 Rhizobia nodulation scoring index

Scoring index used for categorizing root nodulation of *Rhizobium leguminosarum* bv. *trifolii* on all species of *Trifolium.* Based loosely on Corbin *et al.* 1977.

	Description	Level of functionality
0_a	No nodules	None
0_b	Nodules present but all < 1 mm wide and/or lacking pigment, suggesting parasitism	Potentially negative (parasitic)
1	Few nodules, only at distal portions of root system; mostly < 1 mm wide, light pink	Low
2	Nodules scattered throughout root system; many > 1 mm wide, light pink to red	Medium
3	Abundant nodules, particularly in the top 2 mm of root crown; many > 1 mm wide, red to purple indicating presence of the oxygen-carrier leghaemoglobin.	High

C.2 Colonisation by arbuscular mycorrhizal fungi

Differentiation of structure types in the scoring of arbuscular mycorrhizal fungi (AMF). Only arbuscules and vesicles were scored as AMF in the analyses.

Example	Structure type	Description
	Arbuscules	Intracellular branching structures
	Vesicles	Enlarged storage organs located within root cells
	Intracellular hyphae	Any hyphae with extension into root cells, including those characteristic of VAM (e.g. longitudinal hyphae pictured)
	Extracellular fungi	Any fungal structure (e.g. sporangia, spores, hyphae) outside of root
	Root	No fungi present

Appendix D

Mutualist association and plant performance (Chp 2)

D.1 Summary of performance results by *Trifolium* species

Summary information on mutualist association for the seven species of *Trifolium* used in this study.

			Distribution			Performance in glasshouse trials (mean ± S.E.)		
Species	**Common name**	**Years naturalised in NZ***	**Banks Peninsula, NZ populations †**	**Total New Zealand NZMS grids ‡**	**Native range area inhabited x 10^{12} km^2¶**	**Rhizobia nodulation score**	**% AMF colonisation**	**Dry-weight biomass (g)**
T. arvense	Hare's foot clover	138	199	83	26.6	1.6 ± 0.1	32.0 ± 2.0	0.6 ± 0.1
T. campestre	Hop trefoil	147	9	46	20.1	1.8 ± 0.1	20.0 ± 10.0	0.8 ± 0.1
T. glomeratum	Cluster clover	145	564	44	7.7	1.3 ± 0.2	11.1 ± 2.6	1.0 ± 0.1
T. micranthum	Slender trefoil	160	16	23	10.0	1.8 ± 0.2	42.3 ± 6.5	0.8 ± 0.1
T. ornithopodioides	Bird's foot clover	84	5	26	5.4	2.2 ± 0.3	54.8 ± 7.7	0.6 ± 0.1
T. striatum	Knotted clover	138	301	44	10.1	1.6 ± 0.1	28.2 ± 7.8	2.2 ± 0.1
T. tomentosum	Woolly clover	123	30	21	11.4	1.7 ± 0.2	27.5 ± 4.4	1.7 ± 0.2

* Data from Gravuer 2004

† Single-population records based on a 1983-1988 vegetation survey of Banks Peninsula and local environs; nvs.landcareresearch.co.nz; Wiser et al. 2001

‡ Number of 10 x 10 km NZMS260 grids occupied by at least one population; Gravuer 2004

¶ Area estimate (x 10^{12} km^2); Gravuer 2004

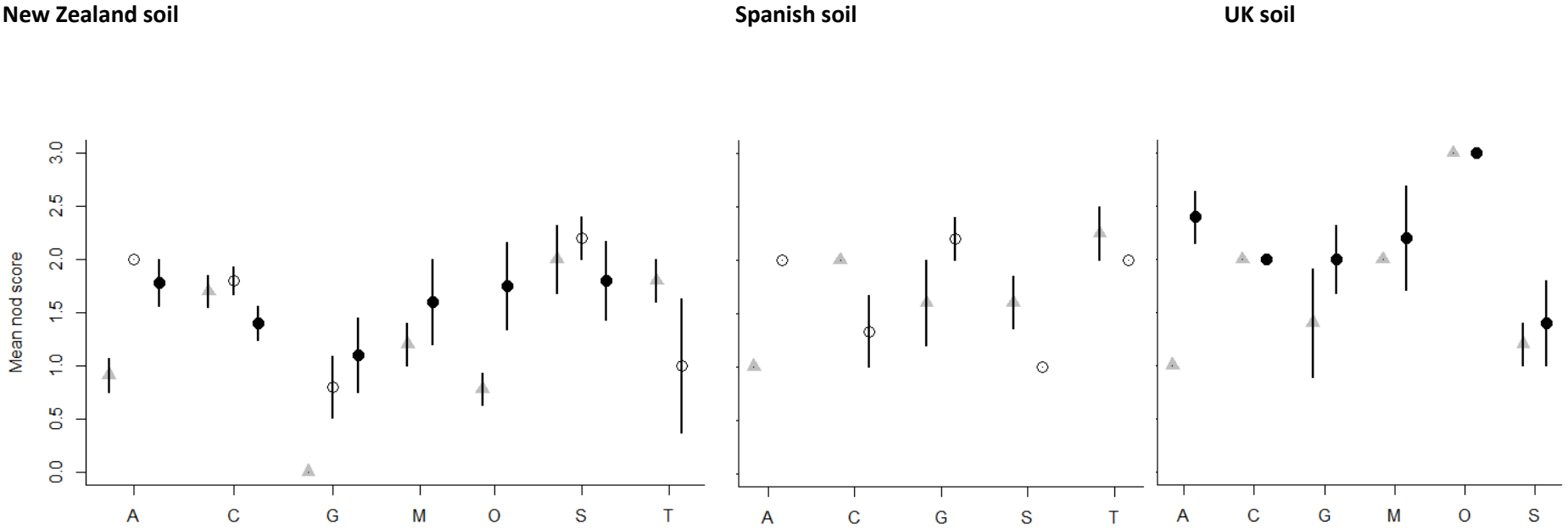
D.2 Floral records of *Trifolium* performance

Recorded size ranges (heights and leaflet dimensions as available) for the seven species of Trifolium used in this study as recorded in the published flora guide in each provenance (New Zealand, Spain and the UK) used in the study and an additional non-native location (California, USA) as a comparison. N/A = data not available.

Species		UK Flora*	Flora Iberica†	Flora of NZ‡	Calif., USA¶
T. arvense	Height:	5–40 cm	3.5–50 cm		
	Leaflets:	10-25 mm	27 x 7 mm	5–20 mm long	5–20 mm
T. campestre	Height:	25–50 cm	2–80 cm		
	Leaflets:	8-10 mm	20 x 12 mm	4–15 mm long	6–15 mm
T. glomeratum	Height:	5–25 cm	3–40 cm		
	Leaflets:	5-10 mm	20 x 15 mm	3–12 mm long	5–12 mm
T. micranthum	Height:	2–20 cm	2–45 cm		
	Leaflets:	~5 mm	8 x 5 mm	2–8 mm long	No Record
T. ornithopodioides	Height:	2–20 cm	2–40 cm		
	Leaflets:	N/A	17 x 8 mm	4–10 mm long	No Record
T. striatum	Height:	5–40 cm	3–60 cm		
	Leaflets:	5-15 mm	27 x 15 mm	5–20 mm long	6–16 mm
T. tomentosum	Height:	N/A (UK	2–35 cm		
	Leaflets:	non-native)	19 x 15 mm	5–10 mm long	4–12 mm

* Ecological Flora of the British Isles, http://www.ecoflora.co.uk
† Flora Iberica (CSIC 2015)
‡ Flora of New Zealand Vol. IV: Naturalised Pteridophytes, Gymnosperms, Dicotyledons (Web et al. 1988)
¶ Jepson Herbarium, University of California, Berkeley; http://ucjeps.berkeley.edu

D.3 Rhizobia nodulation of each *Trifolium* species and seed provenance (Chp 2)

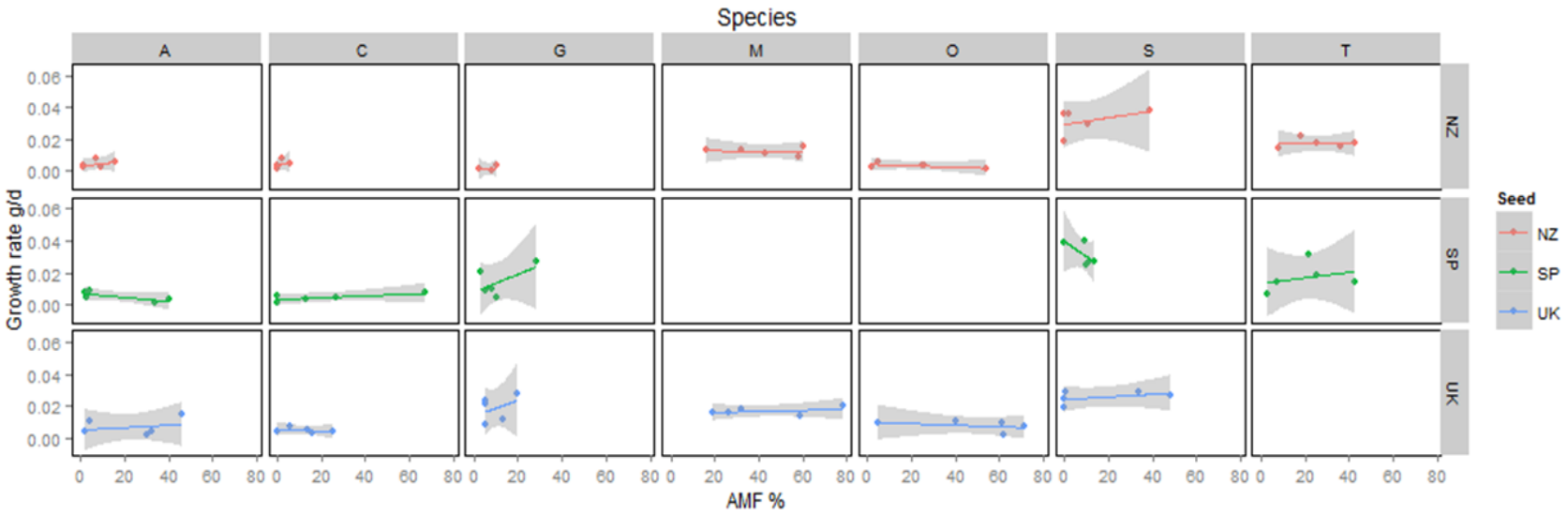


Species codes: A = Trifolium arvense; C = T. campestre; G = T. glomeratum; M = T. micranthum; O= T. ornithopodioides; S = T. striatum; T = T. tomentosum

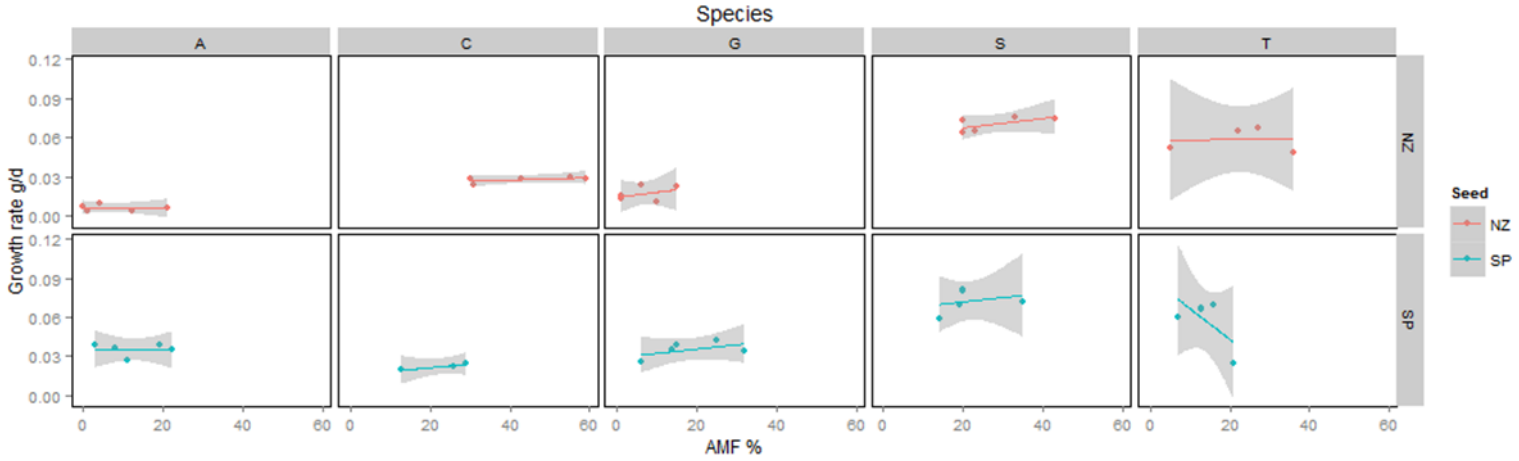
Mean nodulation scores for non-native (New Zealand = ▲) and native (Spain = ○ and UK = ●) provenances of seven species of Trifolium grown in live soils from New Zealand, Spain and the UK. Bars are 95% confidence intervals extracted from the model coefficient tables. Linear mixed-effects models were run separately for each soil type.

D.4 AMF-MB: Growth benefit derived from AMF association for each *Trifolium* species (Chp 2)

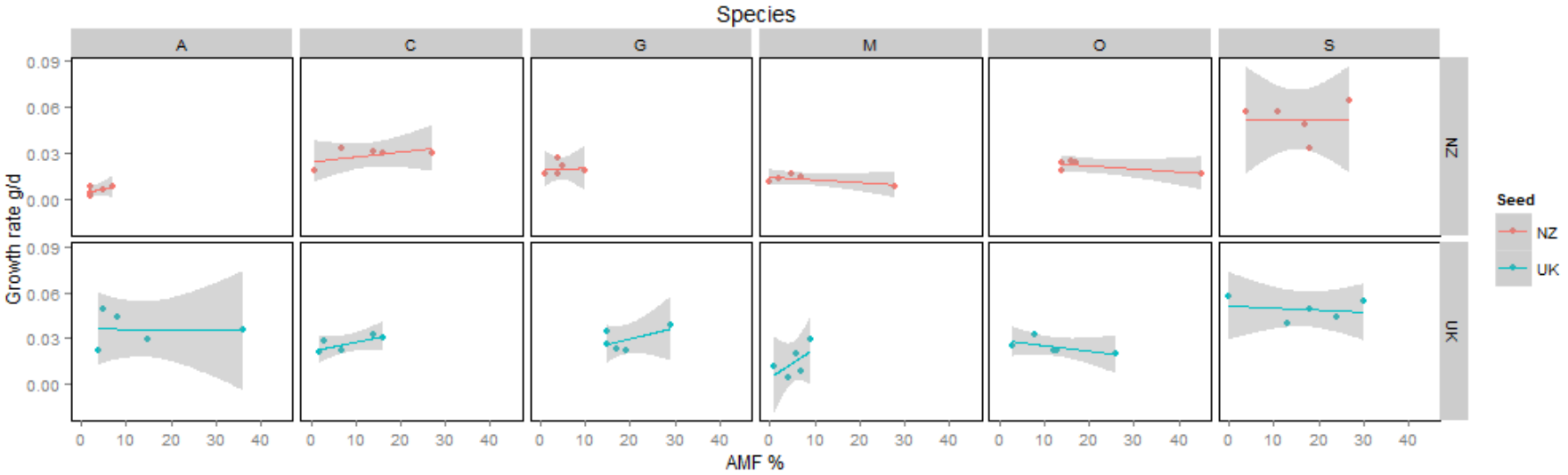
(A) New Zealand soil



(B) Spanish soil



(C) UK soil

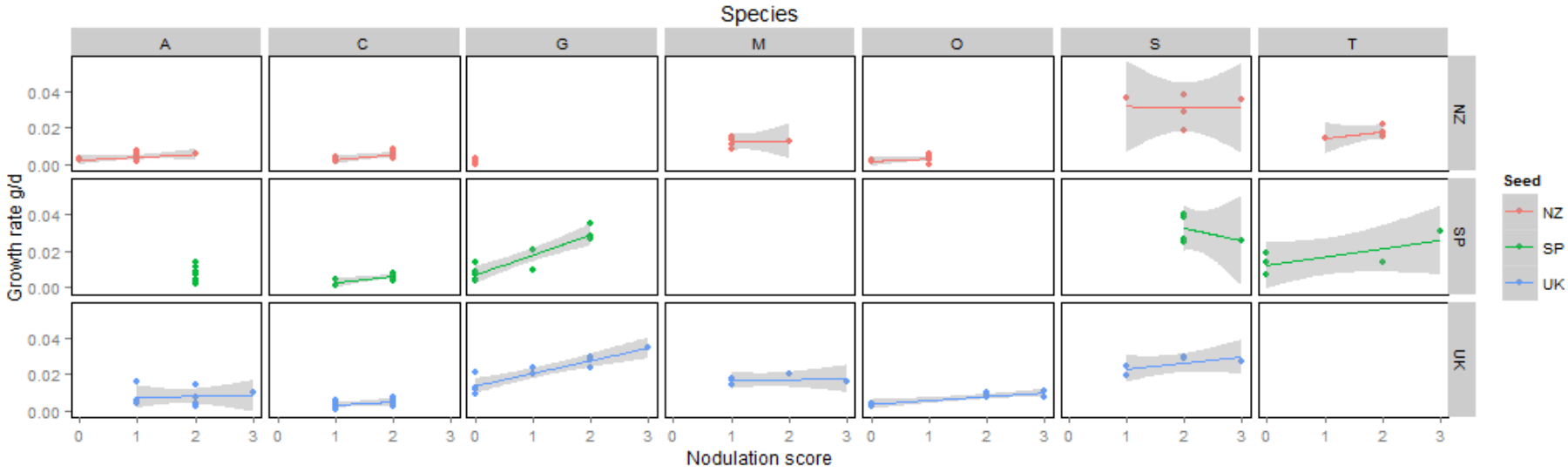


Species codes: A = *Trifolium arvense;* C = *T. campestre;* G = *T. glomeratum;* M = *T. micranthum;* O= *T. ornithopodioides;* S = *T. striatum;* T = *T. tomentosum*

The growth benefit derived from association with arbuscular mycorrhizal colonisation, termed the AMF mutualistic benefit (AMF-MB). Growth rate (dry-weight grams/day) plotted against percentage colonisation by arbuscular mycorrhizal fungi for non-native (top row) and native (Spain, middle row; UK bottom row) provenances of seven species of *Trifolium* grown in unsterilised soil cultivated by conspecifics in (A) New Zealand, (B) Spain and (C) the UK. The shaded region is the 95% confidence interval.

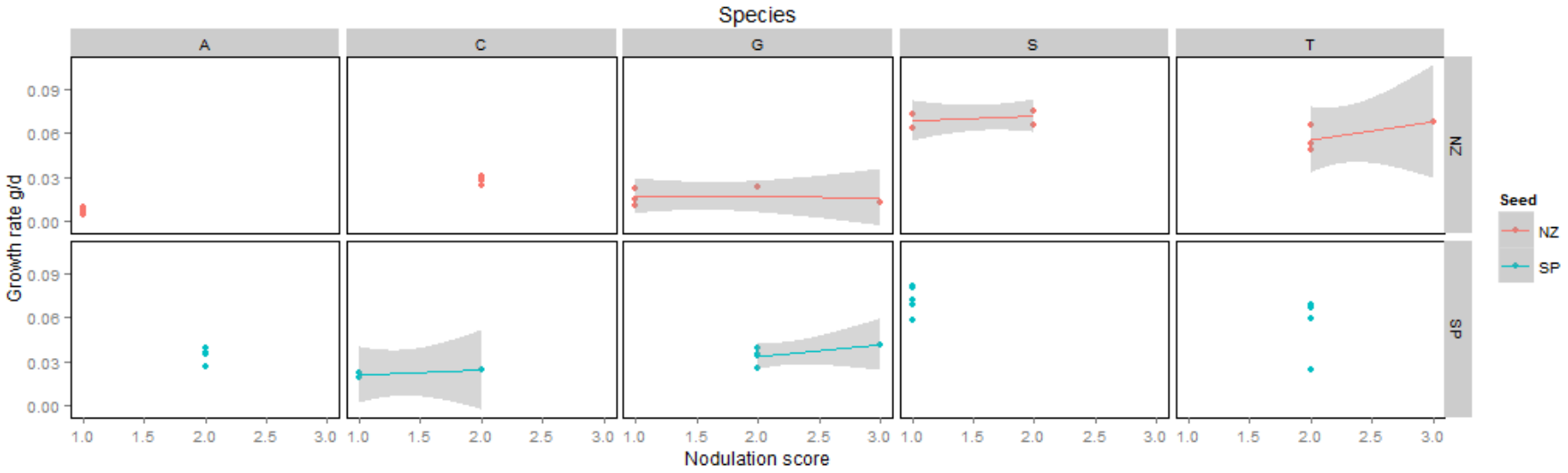
D.5 R-MB: Growth benefit derived from rhizobia association for each *Trifolium* species (Chp 2)

(A) New Zealand soil



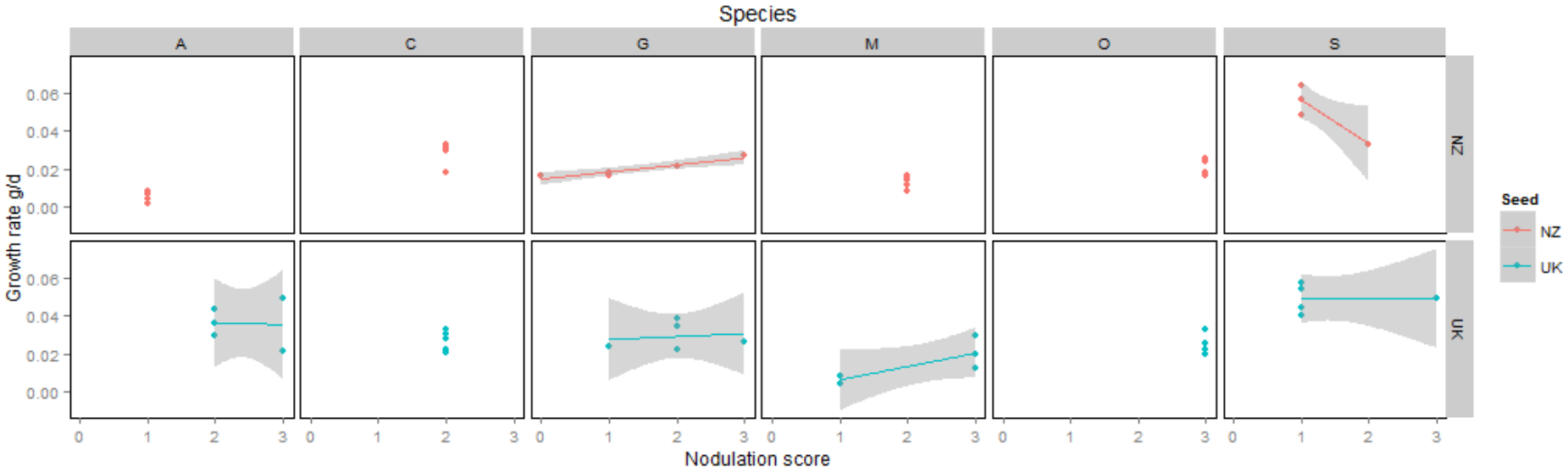
(Figure continues next page)

(B) Spanish soil



(Figure continues next page)

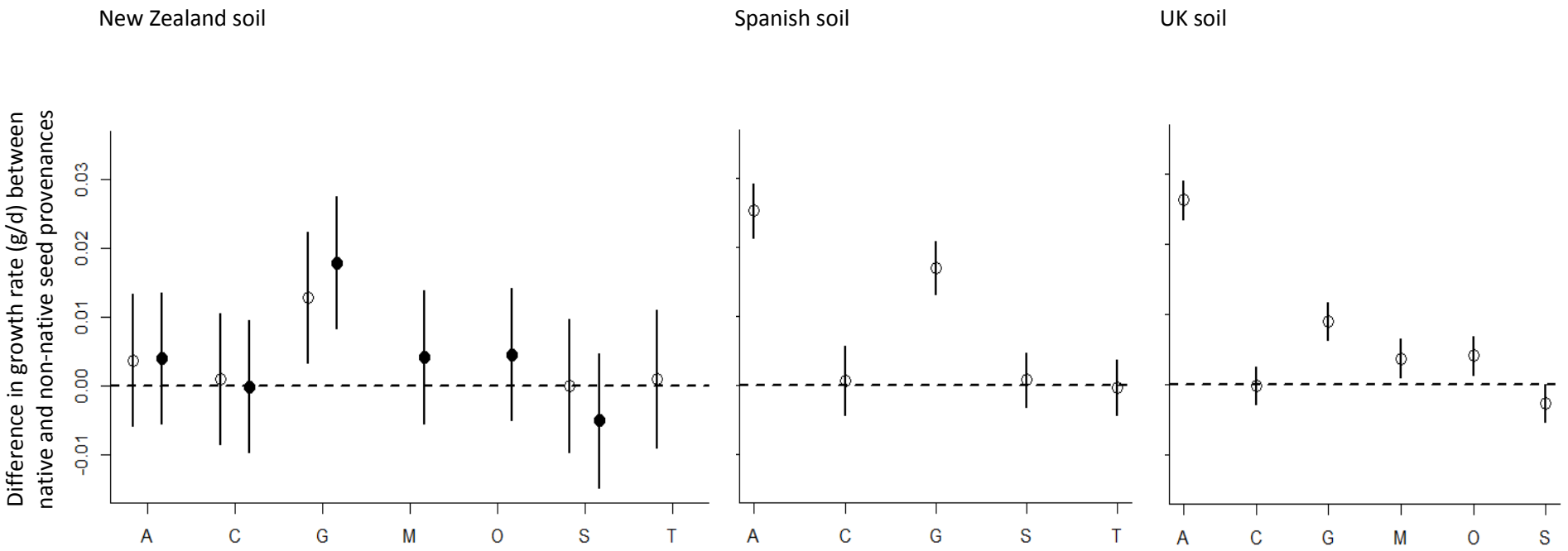
(C) UK soil



Species codes: A = *Trifolium arvense;* C = *T. campestre;* G = *T. glomeratum;* M = *T. micranthum;* O= *T. ornithopodioides;* S = *T. striatum;* T = *T. tomentosum*

The growth benefit derived from association with *Rhizobium leguminosarum* biovar. *trifolii*, termed the rhizobia mutualistic benefit (R-MB).Growth rate (dry-weight grams/day) plotted against percentage nodulation of for non-native (New Zealand, NZ) and native (Spain, SP and UK) provenances of seven species of *Trifolium* grown in unsterilised soil cultivated by conspecifics in (A) New Zealand, (B) Spain and (C) the UK. The shaded region is the 95% confidence interval.

D.6 Interprovenance differences in growth rate for each *Trifolium* species (Chp 2)



Species codes: A = *Trifolium arvense;* C = *T. campestre;* G = *T. glomeratum;* M = *T. micranthum;* O= *T. ornithopodioides;* S = *T. striatum;* T = *T. tomentosum*

Differences in growth rate between non-native (New Zealand) and native (Spain = ○ and UK = ●) provenances of seven species of *Trifolium* grown in unsterilised soils from the non-native range (New Zealand) and native range (Spain or the UK). Points are a reference class in which plants from native provenances are compared to plants from non-native provenances. Thus, zero would represent no difference between provenances and points above the line signify that growth was higher among plants from the native provenance. Bars are 95% confidence intervals extracted from the model coefficient tables. Linear mixed-effects models were run separately for each soil type.

Appendix E

Model codes and statistics tables for mutualism analyses

E.1 R model codes

Table E1. R code for linear mixed-effect (lme) models used to compare the performance (growth rate) and mutualist association (nodulation with rhizobia or colonisation by AMF) of plants from native and non-native seed provenances. Models were fit with the lmer function of the R package "arm" (Gelman & Su 2014) and run separately for each soil provenance (New Zealand (nzdat), Spain (spdat) and the UK (ukdat). Models were run in pairs with and without the fixed factor "seed" and each pair of models analysed via ANOVA to extract a significance value for seed.

Model	R code
Log-transformed growth rate as a factor of seed provenance	m <- lmer(log(biom) ~ seed + (1|ref), data=dat)
Colonisation by AMF, controlling for nodulation	m <-lmer(amf~seed + nods2 + (1|ref), data=dat)
Nodulation with rhizobia as a factor of seed provenance	m<-lmer(nods2~seed + (1|ref), data=dat)
Benefit of rhizobia (R-MB) as a factor of growth rate with seed as a fixed factor	m <- lmer(biom~nods2 + seed + (1|ref), data=dat
Benefit of AMF (AMF-MB) as a factor of growth rate with seed as a fixed factor	m <- lmer(biom~amfl + seed + (1|ref), data=dat
Shoot:root ratio as a factor of seed provenance	m<- lmer(srratio~seed + (1|ref), data=dat)

Variable	Purpose	R code
ref	Make species and site random variables	ref <- factor(paste(dat$species, dat$site))
amfl	% AMF colonisation, logit-transformed	amfl <- logit(dat$amfp, percents=max(dat$amfp, na.rm = TRUE) > 1, adjust)

E.2 "Tripartite" association ANOVA

Results of the ANOVA models to investigate the predicted "tripartite" relationship between rhizobia ("nods2," a 0-3 scale) and arbuscular mycorrhizal fungi ("amfl," a logit-transformed percentage). "Ref" is a random reference variable that includes the effects of species and site. Analyses were run separately for each soil, (1) New Zealand, (2) Spain and (3) UK. Regression plots are located in Appendix E.3.

(1) NZ soil

aov(amfl~nods2 + amfl:nods2 + amfl:nods2:seed + (Error(ref)), data=nzdat))

```
Error: ref
                Df Sum Sq Mean Sq F value   Pr(>F)
nods2            1   1.16    1.16   1.249    0.273
seed             2  53.92   26.96  28.981 1.51e-07 ***
amfl:nods2       1 113.86  113.86 122.399 9.88e-12 ***
amfl:nods2:seed  2   0.06    0.03   0.030    0.970
Residuals       28  26.05    0.93
```

(2) Spanish soil

aov(amfl~nods2 + amfl:nods2 + amfl:nods2:seed + (Error(ref)), data=spdat)

```
Error: ref
                Df Sum Sq Mean Sq F value   Pr(>F)
nods2            1   0.06    0.06   0.726 0.404238
seed             1   2.45    2.45  29.884 2.37e-05 ***
amfl:nods2       1  45.23   45.23 552.737 4.83e-16 ***
amfl:nods2:seed  1   1.47    1.47  17.963 0.000403 ***
Residuals       20   1.64    0.08
```

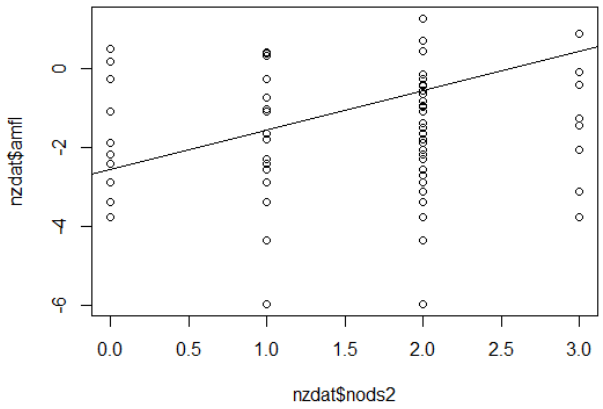
(3) UK soil

aov(amfl~nods2 + amfl:nods2 + amfl:nods2:seed + (Error(ref)), data=ukdat)

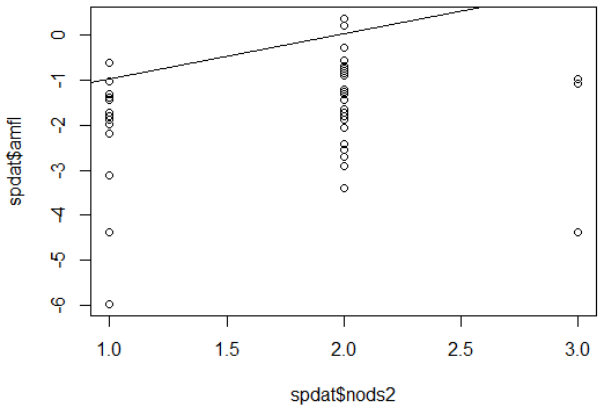
```
Error: ref
                Df Sum Sq Mean Sq F value   Pr(>F)
nods2            1   1.07    1.07   4.026   0.0553 .
amfl:nods2       1  47.05   47.05 176.235 4.33e-13 ***
amfl:nods2:seed  1   0.16    0.16   0.607   0.4428
Residuals       26   6.94    0.27


Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

E.3 “Tripartite” association regressions

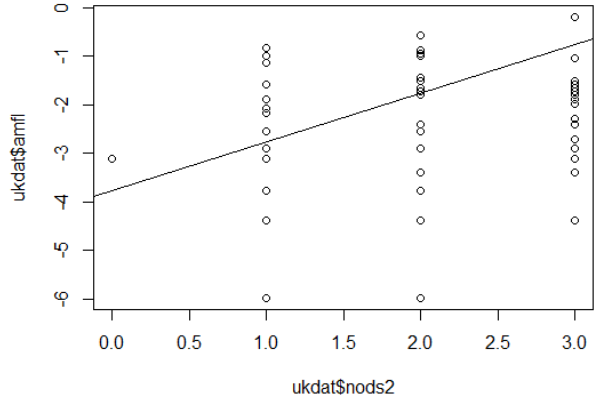
(A) New Zealand soil



(B) Spanish soil



(C) UK soil



Regressions for the investigation of “tripartite” association between arbuscular mycorrhizal fungi (AMF) and rhizobia. AMF data are percentage colonisation, logit-transformed and rhizobia nodulation is a score of 0 to 3 (Appendix C.1 for scoring details). Analyses were separated by soil location. Full ANOVA results are in Appendix E.2.

E.4 Mutualist-association and mutualist-benefit ANOVAs

Results of the ANOVAs used to analyse the significance of the fixed factor seed provenance ("seed") using the paired linear mixed effects models explained in Appendix Table E1. Five outputs are shown: (A) arbuscular mycorrhizal colonization (B) rhizobia nodulation, (C) Mutualistic benefit of rhizobia (R-MB); (D) Mutualistic benefit of AMF (AMF-MB); and (E) Shoot-root ratios ("srratio"). Each P value (bolded) provides the significance of "seed provenance" in in the analysis performed each soil location: [1] New Zealand soil, [2] Spanish soil and [3] UK soil.

(A) Arbuscular mycorrhizal colonisation

[[1]] NZ soil

```
m11a: amfl ~ 1 + nods2 + (1 | ref), m11: amfl ~ seed + nods2 + (1 | ref)
     Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m11a  4 365.38 375.34 -178.69   357.38
m11   6 366.15 381.08 -177.08   354.15 3.2311      2     0.1988
```

[[2]] Spanish soil

```
m12a: amfl ~ 1 + nods2 + (1 | ref), m12: amfl ~ seed + nods2 + (1 | ref)
     Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m12a  4 150.27 157.67 -71.134   142.27
m12   5 151.61 160.86 -70.803   141.61 0.6613      1     0.4161
```

[[3]] UK soil

```
m13a: amfl ~ 1 + nods2 + (1 | ref), m13: amfl ~ seed + nods2 + (1 | ref)
     Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m13a  4 193.89 202.27 -92.944   185.89
m13   5 195.48 205.95 -92.738   185.48 0.4119      1      0.521
```

(B) Rhizobia nodulation

[[1]] NZ soil

```
m1a: nods2 ~ 1 + (1 | ref), m1: nods2 ~ genotype + (1 | ref)
    Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m1a  3 348.75 357.55 -171.37   342.75
m1   4 339.07 350.81 -165.54   331.07 11.677      1  0.0006327 ***
```

[[2]] Spanish soil

```
m2a: nods2 ~ 1 + (1 | ref), m2: nods2 ~ genotype + (1 | ref)
    Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m2a  3 88.008 93.559 -41.004   82.008
m2   4 89.796 97.196 -40.898   81.796 0.2127      1     0.6447
```

[[3]] UK soil

```
m3a: nods2 ~ 1 + (1 | ref), m3: nods2 ~ genotype + (1 | ref)
    Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m3a  3 149.04 155.32 -71.519   143.04
m3   4 145.40 153.77 -68.698   137.40 5.6416      1    0.01754 *
```

(E.4 cont.)

(C) Mutualistic benefit of rhizobia (growth as a factor of nodulation level)

[[1]] NZ soil

```
m1a: biom ~ nods2 + (1 | ref), m1: biom ~ nods2 + seed + (1 | ref)
    Df     AIC     BIC logLik deviance  Chisq Chi Df Pr(>Chisq)
m1a  4 -1000.0 -988.27 504.01  -1008.0
m1   6 -1002.8 -985.17 507.39  -1014.8 6.7651      2    0.03396 *
```

[[2]] Spanish soil

```
m2a: biom ~ nods2 + (1 | ref), m2: biom ~ nods2 + seed + (1 | ref)
    Df     AIC     BIC logLik deviance  Chisq Chi Df Pr(>Chisq)
m2a  4 -233.39 -225.99 120.69  -241.39
m2   5 -240.83 -231.58 125.41  -250.83 9.4385      1   0.002125 **
```

[[3]] UK soil

```
m3a: biom ~ nods2 + (1 | ref), m3: biom ~ nods2 + seed + (1 | ref)
    Df     AIC     BIC logLik deviance  Chisq Chi Df Pr(>Chisq)
m3a  4 -341.82 -333.44 174.91  -349.82
m3   5 -344.14 -333.67 177.07  -354.14 4.3259      1    0.03754 *
```

(D) Mutualistic benefit of AMF (growth as a factor of logit-transformed % colonisation)

[[1]] NZ soil

```
m1a: biom ~ amfl + (1 | ref), m1: biom ~ amfl + seed + (1 | ref)
    Df     AIC     BIC logLik deviance Chisq Chi Df Pr(>Chisq)
m1a  4 -606.83 -596.87 307.42  -614.83
m1   6 -606.76 -591.82 309.38  -618.76 3.926      2     0.1404
```

[[2]] Spanish soil

```
m2a: biom ~ amfl + (1 | ref), m2: biom ~ amfl + seed + (1 | ref)
    Df     AIC     BIC logLik deviance  Chisq Chi Df Pr(>Chisq)
m2a  4 -243.33 -235.93 125.67  -251.33
m2   5 -250.02 -240.77 130.01  -260.02 8.6856      1   0.003207 **
```

[[3]] UK soil

```
m3a: biom ~ amfl + (1 | ref), m3: biom ~ amfl + seed + (1 | ref)
    Df     AIC     BIC logLik deviance  Chisq Chi Df Pr(>Chisq)
m3a  4 -343.25 -334.88 175.63  -351.25
m3   5 -346.87 -336.40 178.43  -356.87 5.6154      1     0.0178 *
```

(E.4 cont.)

(E) Shoot:root ratio

[[1]] NZ soil

```
m1: srratio ~ 1 + (1 | ref), m1a: srratio ~ seed + (1 | ref)
    Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m1   3 698.97 707.77 -346.49   692.97
m1a  5 698.98 713.65 -344.49   688.98 3.9943      2     0.1357
```

[[2]] Spanish soil

```
m2: srratio ~ 1 + (1 | ref), m2a: srratio ~ seed + (1 | ref)
    Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m2   3 106.08 111.63 -50.041  100.081
m2a  4 107.43 114.83 -49.713   99.427 0.6543      1     0.4186
```

[[3]] UK soil

```
m3: srratio ~ 1 + (1 | ref), m3a: srratio ~ seed + (1 | ref)
    Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m3   3 175.22 181.50 -84.607   169.22
m3a  4 171.57 179.94 -81.783   163.57 5.6488      1    0.01747 *
```

Appendix F

Model codes and statistics tables for flavonoid analyses (Chp 3)

F.1 R model codes

Table F1. R code for linear mixed-effect (lme) models used to compare flavonoid richness and concentration of plants from native and non-native seed provenances. Models were fit with the lmer function of the R package arm (Gelman & Su 2014) and run separately in each soil origin [New Zealand, Spain and the UK] and in each treatment [sterile or live]. Models were run in pairs with and without the fixed factor "seed" and each pair of models analysed via ANOVA to extract a significance value for seed.

Model	R code
Log-transformed concentration (ppm) all flavonoids (sterilised soil)	m <-lmer(log(t.ppm)~seed + (1|ref), data=dat.sterile)
Log-transformed concentration (ppm) all flavonoids (unsterilised soil)	m <-lmer(log(t.ppm)~seed + (1|ref), data=dat.live)
Logit-transformed richness (number of all flavonoid peaks) (sterilised soil)	m <-lmer(total.peaks~seed + (1|ref) + (1|od), data=dat.sterile, family=poisson)
Logit-transformed richness (number of all flavonoid peaks) (unsterilised soil)	m <-lmer(total.peaks~seed + (1|ref) + (1|od), data=dat.live, family=poisson)
Log-transformed concentration (ppm) daidzein (sterilised soil)	m <-lmer(log(t.ppm.daidzein)~seed + (1|ref), data=dat.sterile)
Log-transformed concentration (ppm) daidzein (unsterilised soil)	m <-lmer(log(t.ppm.daidzein)~seed + (1|ref), data=dat.live)
Logit-transformed richness (number of all flavonoid peaks) (sterilised soil)	m <-lmer(peaks.daidzein~seed + (1|ref) + (1|od), data=dat.sterile, family=poisson)
Logit-transformed richness (number of all flavonoid peaks) (unsterilised soil)	m <-lmer(peaks.daidzein~seed + (1|ref) + (1|od), data=dat.live, family=poisson)

Variable	Purpose	R code
ref	Groups species and site as random variables	ref <- factor(paste(flav$species, flav$site))
(1|od)	A random effect that takes a value between 1 and "N" (the total number of observations) to control for over-dispersion in count data	od <- (1:N)

F.2 Flavonoid-biomass trade-off ANOVA

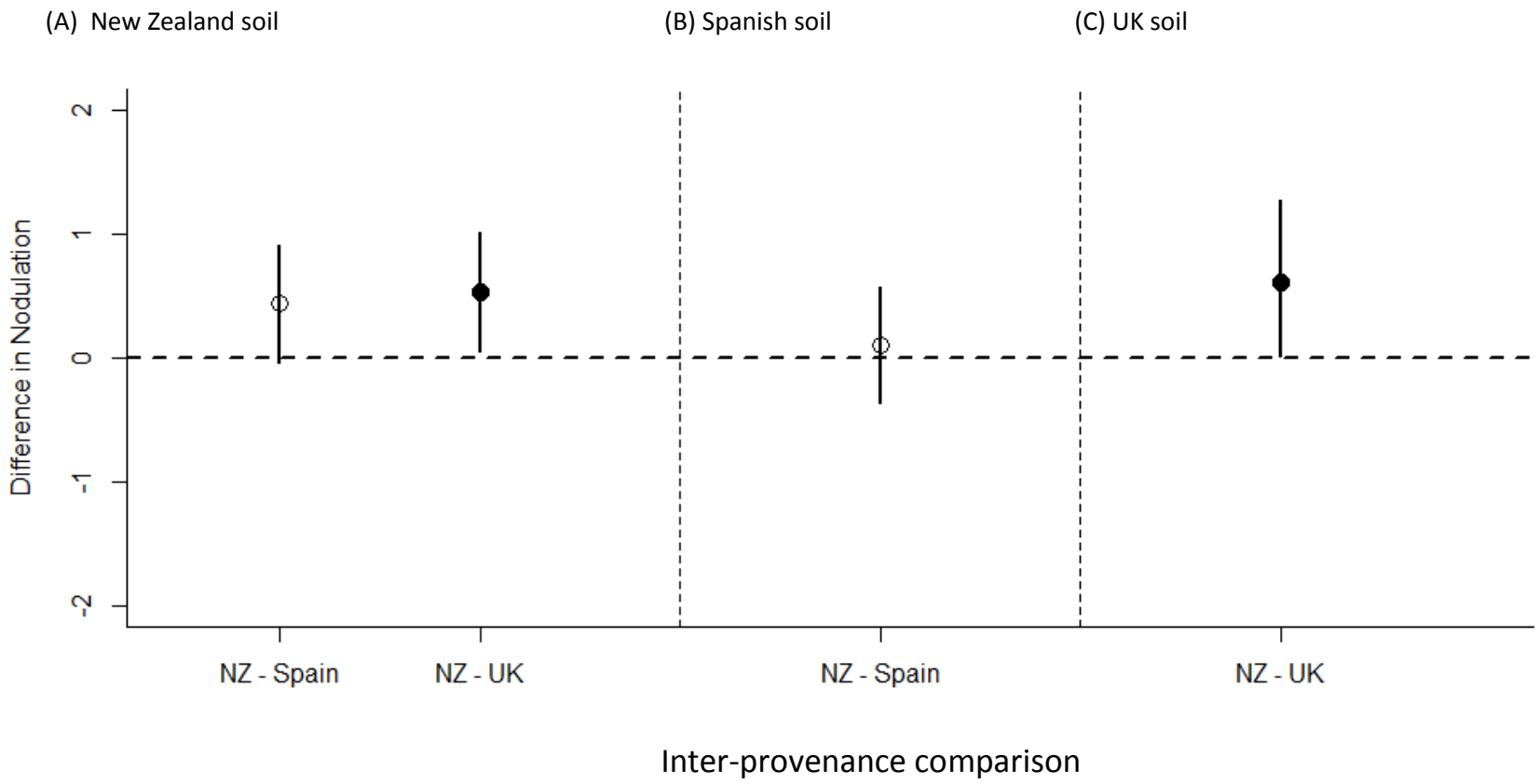
Table F2. Results of the linear mixed-effects model analysis of flavonoid concentration (log transformed in ppm) as a factor of root biomass (log transformed in grams) with seed provenance (New Zealand [seedNZ], Spain [seedSP] and the UK [seedUK] designated as the fixed factor to test whether native and non-native seed provenances differ in their relative allocation to flavonoids and root biomass. Data is for all soil treatments combined. Results are detailed in panel B of Figure 3.5.

Model code: lm(formula = log(t.ppm) ~ (seed/log(b.biomass) + 0), data = flav)

Residuals:	Min = -1.91454	1Q = -0.35543	Median = 0.02383	3Q = 0.38528	Max = 1.15095
Variable	**Coefficients**	**Estimate Std. Error**	**t value**	**Pr(>|t|)**	
seedNZ	2.68977	0.10664	25.223	<2e-16 ***	
seedSP	2.79860	0.11472	24.395	<2e-16 ***	
seedUK	3.16954	0.14702	21.558	<2e-16 ***	
seedNZ:log(b.biomass)	-0.09235	0.04526	-2.041	0.0425 *	
seedSP:log(b.biomass)	-0.16359	0.06158	-2.656	0.0085 **	
seedUK:log(b.biomass)	0.06004	0.07903	0.760	0.4483 (NS)	

Residual standard error: 0.5641 on 213 degrees of freedom
Multiple R-squared: 0.9664, Adjusted R-squared: 0.9654
F-statistic: 1020 on 6 and 213 DF, p-value: < 2.2e-16

F.3 Inter-provenance differences in rhizobia nodulation (Chp 3)



Mean difference in nodulation score between non-native (New Zealand) and native (Spain = ○ and UK = ●) provenances of five species of *Trifolium* grown in unsterilised rhizosphere soils cultivated *in situ* by conspecifics in the non-native range (New Zealand, NZ) and native range (Spain and the UK). Points are a reference class in which plants from native provenances are compared to plants from non-native provenances. Thus, zero would represent no difference between provenances and points above the line signify that nodulation was higher among plants from the native provenance. Nodulation was measured via a 0-3 nodulation score; see Appendix C.1 for scoring details). Bars are 95% confidence intervals extracted from the model coefficient tables. Linear mixed-effects models were run separately by soil.

F.4 Summary information for the five species of *Trifolium*

Summary information for the five species of *Trifolium* used in the flavonoid analyses (Chp 3). Values are means ± S.E.

Species	Years NZ naturalised*	Flavonoid production				Rhizobia nodulation score	Dry-weight biomass (g)
		Richness (number of flavonoids)	Flavonoid concentration (ppm)	Daidzein richness (number of peaks with >85% similarity to daidzein standard)	Daidzein concentration (ppm)		
T. arvense	138	13.2 ± 0.7	30.2 ± 1.7	2.1 ± 0.1	7.3 ± 0.7	1.6 ± 0.1	0.6 ± 0.1
T. glomeratum	145	10.3 ± 0.4	26.4 ± 2.4	1.7 ± 0.3	5.1 ± 1.0	1.3 ± 0.2	1.0 ± 0.1
T. ornithopodioides	84	11.0 ± 0.4	17.8 ± 1.9	3.4 ± 0.2	12.2 ± 1.6	2.2 ± 0.3	0.6 ± 0.1
T. striatum	138	16.2 ± 0.5	20.2 ± 1.5	3.9 ± 0.2	14.9 ± 1.3	1.6 ± 0.1	2.2 ± 0.1
T. tomentosum	123	12.8 ± 0.4	17.6 ± 1.2	4.1 ± 0.1	13.1 ± 1.2	1.7 ± 0.2	1.7 ± 0.2
Averages across species		12.9 ± 0.3	23.2 ± 0.9	3.0 ± 0.1	10.3 ± 0.6		

* Data from Gravuer 2004

F.5 Flavonoid concentration and richness by seed provenance and soil

Flavonoid concentration and richness among plants from native (Spain and the UK) and non-native (New Zealand) provenances grown in (A) sterilised or (B) unsterilised soils from each provenance. P values are the significance of seed origin and were extracted from the linear mixed-effects models.

			Flavonoid richness			Flavonoid concentration			Daidzein richness			Daidzein concentration		
Soil origin	Soil	Seed origin	Mean no. compounds	S.E.	*P*	Mean ppm	S.E.	*P*	Mean no. compounds	S.E.	P	Mean ppm	S.E.	*P*
(A) Sterilised														
NZ	S	NZ	12.8	± 0.9	NS 0.34	22.3	± 2.9	NS 0.19	3.0	± 0.5	NS 0.70	8.3	± 1.4	NS 0.06
NZ	S	Spain	12.8	± 0.8		26.4	± 2.9		2.9	± 0.5		8.8	± 1.6	
NZ	S	UK	11.3	± 0.9		19.6	± 2.9		2.4	± 0.5		5.4	± 1.3	
Spain	S	NZ	10.7	± 1.1	Sig= .003	18.3	± 1.9	NS 0.08	3.2	± 0.4	NS 0.21	12.7	± 2.1	NS 0.07
Spain	S	Spain	16	± 1.2		26.5	± 3.0		4.1	± 0.2		18.0	± 2.1	
UK	S	NZ	10.1	± 1.1	Sig< .001	19.7	± 2.5	Sig= 0.03	3.3	± 0.3	NS 0.64	12.8	± 1.4	Sig= 0.03
UK	S	UK	16.5	± 1.4		28.8	± 4.4		3.6	± 0.2		18.5	± 3.0	

(F.5 continues next page)

			Flavonoid richness			Flavonoid concentration			Daidzein richness			Daidzein concentration		
Soil origin	Soil	Seed origin	Mean no. compounds	S.E.	*P*	Mean ppm	S.E.	*P*	Mean no. compounds	S.E.	P	Mean ppm	S.E.	*P*
(B) Unsterilised														
NZ	U	NZ	13.1	± 0.8		23.8	± 3.3		2.3	± 0.4		5.8	± 1.1	
NZ	U	Spain	13.2	± 0.9	NS 0.76	28.2	± 3.8	NS 0.18	2.3	± 0.4	NS 0.80	5.8	± 1.0	NS 0.16
NZ	U	UK	13.7	± 0.8		28.4	± 3.6		2.4	± 0.4		9.5	± 2.7	
Spain	U	NZ	11.7	± 1.7	NS	15.9	± 2.4		2.9	± 0.4		10.1	± 2.2	
Spain	U	Spain	13	± 1.1	0.32	17.6	± 2.6	NS 0.37	3.6	± 0.2	NS 0.36	11.2	± 1.4	NS 0.16
UK	U	NZ	10.8	± 1.2		19.1	± 3.3		3.4	± 0.4		11.5	± 2.1	
UK	U	UK	14.8	± 1.2	Sig= .007	24.1	± 3.6	NS 0.10	4.0	± 0.2	NS 0.47	16.0	± 3.4	NS 0.20

F.6 Daidzein-rhizobia correlations

Correlations between daidzein richness (A) or concentration (B) and rhizobia nodulation for each of the unsterilised soils (all soils combined, NZ soils, Spanish soils and UK soils). Pearson's correlation coefficient defined as slight (0.2-0.3), moderate (0.4-0.5), strong (0.6-0.7), very strong (0.8-1.0).

Treatment	Nod mean ± SE	Pearson's score	df	t-value	95% C.I.	P value	Correlation
(A) Daidzein richness							
All soils combined	1.59 ± 0.08	0.5134	111	6.3031	0.3631; 0.6376	6.069e-09 **	Strong, +
New Zealand	1.38 ± 0.13	0.4535	61	3.9737	0.2317; 0.6304	0.0001897 **	Moderate, +
Spain	1.81 ± 0.12	0.4978	24	2.8118	0.1368; 0.7421	0.00966 **	Moderate, +
UK	1.92 ± 0.19	0.4868	22	2.6136	0.1037; 0.7440	0.01586 *	Moderate, +
(B) Daidzein concentration							
All soils combined	1.59 ± 0.08	0.2955	111	3.2593	0.1172; 0.4554	0.001483 **	Slight, +
New Zealand	1.38 ± 0.13	0.3874	61	3.2822	0.1545; 0.5796	0.001707 **	Slight, +
Spain	1.81 ± 0.12	-0.1356	24	-0.6706	-0.4968; 0.2657	0.5089 NS	None
UK	1.92 ± 0.19	-0.1160	22	-0.5477	-0.4962; 0.3015	0.5894 NS	None

Appendix G

Details of the HPLC method (Chp 3)

G.1 HPLC equipment and settings

High-performance liquid chromatography (HPLC) analyses were performed on an Agilent 1100 series HPLC machine using a C18 4.6 x 150 mm Kinetex column for separation. Methodology was optimised during pre-trials to enable full separation of flavonoids for all five species of *Trifolium* used in the study.

Flow rate of the mobile phase was 0.5 mL/min. Column temperature was set to 40° C.

Samples were run using the following five-gradient time step for a total run time of 30 min.

Time (in min)	Solvent A: 0.01% acetic acid in Milli-Q water	Solvent B: 0.01% acetic acid in Acetonitrile
0	95%	5%
9	70%	30%
10	60%	40%
11	60%	40%
21	40%	60%
22	95%	5%
30	95%	5%

Appendix H

Supplementary data for competition analyses (Chp 4)

H.1 R model code

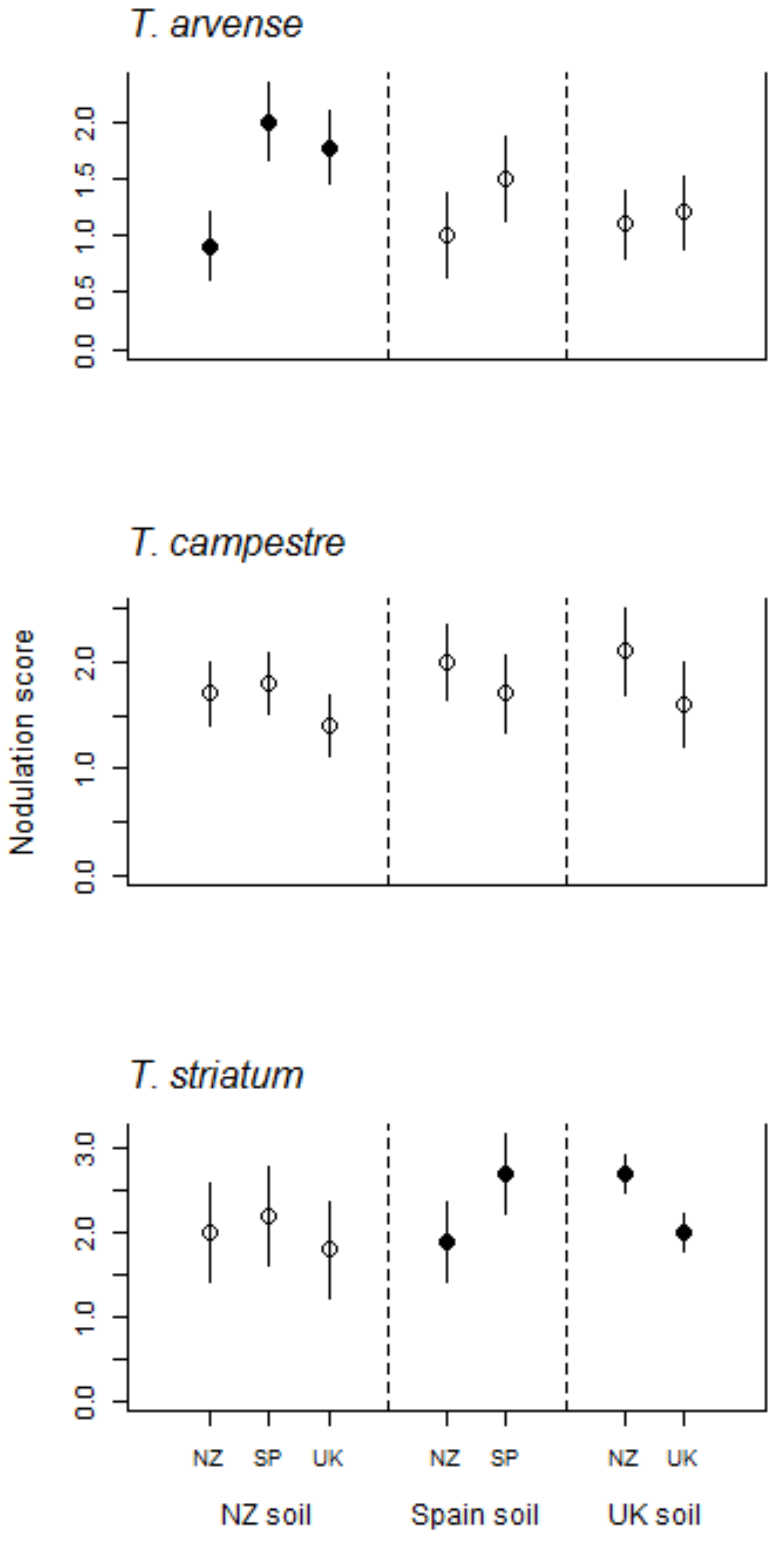
R code for linear mixed-effect (lme) models used to compare (A) single plant performance (growth rate) and (B) competitive ability (RCI value) of plants from native and non-native seed provenances. Models were fit with the lmer function of the R package "arm" (Gelman & Su 2014), run separately for each soil. (A) Models to analyse differences in growth between native and non-native plants in non-competitive conditions (single plant treatments) single-plant was run with (m1-m3) and without (m1a-m3a) the fixed factor "seed" and the pair of models was analysed via ANOVA. (B) Competition models were used to extract the mean growth rate and associated uncertainty for each seed provenance and seed-provenance combination having accounted for site effects. Mean growth rates and their uncertainties were used to calculate RCI indices via a simulation approach.

Model	R code
(A) Growth analysis (singly-grown plants only)	
Log-transformed growth rate as a factor of seed provenance in NZ soil	m1 <- lmer(log(biom) ~ seed + (1|site)+nods2, data=as, subset=soil=="NZ"
*m1 with "seed" removed	m1a <- lmer(log(biom) ~ 1 + (1|site)+nods2, data=as, subset=soil=="NZ"
Log-transformed growth rate as a factor of seed provenance in Spanish soil	m2 <- lmer(log(biom) ~ seed + (1|site)+nods2, data=as, subset=soil=="SP"
*m2 with "seed" removed	m2a <- lmer(log(biom) ~ 1 + (1|site)+nods2, data=as, subset=soil=="SP"
Log-transformed growth rate as a factor of seed provenance in UK soil	m3 <- lmer(log(biom) ~ seed + (1|site)+nods2, data=as, subset=soil=="UK"
*m3 with "seed" removed	m3a <- lmer(log(biom) ~ 1 + (1|site)+nods2, data=as, subset=soil=="UK"
(B) Competition analysis (single vs paired growth)	
Log-transformed growth rate as a factor of group in NZ soil	m4<- lmer(log(biom) ~ group-1 + (1|site), data=as, subset=soil=="NZ")
Log-transformed growth rate as a factor of group in Spanish soil	m5<- lmer(log(biom) ~ group-1 + (1|site), data=as, subset=soil=="SP")
Log-transformed growth rate as a factor of group in UK soil	m6<- lmer(log(biom) ~ group-1 + (1|site), data=as, subset=soil=="UK")

(H.1 cont.)

Model variable	Purpose
seed	A fixed factor to test for differences in performance between seed provenances
site	A random factor to control for differences between the five soil replicates in each soil (NZ, Spain, UK)
nods2	A fixed factor to control for the effect of rhizobia colonisation on growth rate
group	A variable grouping plant origin (NZ, Spain, UK) pot treatment (single vs. paired) and the origin of the competitor plant (NZ, Spain, UK)

H.2 Inter-provenance difference in rhizobia nodulation (Chp 4)



Differences in rhizobia nodulation score between native (Spain and UK) and non-native seed provenances in the competition paired-pot treatments. Filled circles represent significant differences between the non-native and native provenances. Differences in growth related to nodulation were controlled in the linear mixed-effects models in Appendix H.1.